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REMARKS

Claims 1, 4-18, 20, 22, 24, and 27-31 were pending. Claims 1, 17, 20, and 28 are amended herein. Support for these amendments is found throughout the specification, e.g., at ¶26. It is believed no new matter has been added. No claim is allowed.

Formal Matters

Applicant gratefully acknowledges the entry of the Amendment dated September 24, 2003 and the resultant withdrawal of the objection to the oath/declaration, the objection to claim 26 under 37 C.F.R. § 1.75, and the rejection of claims 19-27 under 35 U.S.C. § 112, second paragraph.

Applicant notes that while claims 17 and 28 are included as rejected in the Office Action summary, these claims are not included in any specific rejection under the cited references. Thus, Applicant has rewritten these claims as independent claims and respectfully request that these claims be indicated as free of prior art.

Rejection Under 35 U.S.C. § 103 (a)

Claims 1, 4, 6-7, 10-12, 14-15, 18, 20, 22, and 29-30 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Jakobovits et al. in view of Edelman et al. (*Meth. Enzymol.*) and as necessary Edelman et al. (U.S. Patent No. 3,843,324) for reasons of record. Claim 8, 16, 24, and 27 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Jakobovits et al. in view of Edelman et al (both references) as applied to claims 1, 20, and 22 above, and further in view of Chang (WO 84/03151) for reasons of record. Claim 9 is rejected as unpatentable under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Jakobovits et al. in view of Edelman et al (both references) as applied to claim 1, and further in view of Kupchik for reasons of record. Claim 13 is rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Jakobovits et al. in view of Edelman et al (both references) as applied to claims 1, and further in view of Seifert (U.S. Patent 5,721,120) for reasons of record. Applicant traverses these rejections.

Applicant respectfully submits that there is no suggestion or motivation to modify Jakobovits or combine its teachings with those of Edelman, Chang, Kupchik, and Seifert. Applicant

notes that the mere fact that the reference can be combined or modified is not enough to render the resultant combination obvious. The references themselves must suggest the desirability of the combination or modification. *See* MPEP § 2143.01.

First, Jakobovits fails to provide more than an invitation to try to extend its observations using lectin/oligosaccharide interactions on enzymatically treated red blood cells. Jakobovits describes cell surface receptors at only two places. First, Jakobovits makes an initial statement regarding the state of the art regarding the isolation of cell surface receptors for different classes of effectors such as hormones, toxins, antigens, and lectins. Next, Jakobovits concludes their report by describing potential applications for the disclosed technique. In this paragraph, Jakobovits discloses that they are applying the isolation technique to a different cell population, *i.e.*, lymphocytes, for the isolation of lectin receptors. This statement without more does not indicate to the skilled artisan whether or not this technique will be successful in this as yet untested population. It is, in fact, simply an articulation of an intent to try the technique without indicating any reasonable expectation of success. The very fact that Jakobovits is now exploring its feasibility in nucleated cell types using non-lectin receptors suggests that additional experimentation is required before general applicability can be established.

Moreover, it is well known in the art that lectins are complex molecules that typically can interact with multiple cell surface oligosaccharides simultaneously. Thus, it is generally not assumed that lectin-cell surface receptor interactions predict antigen, hormone, or toxin interactions with a single discrete cell surface receptor. This is particularly true when the lectin binds the target cell population only after enzymatic surface treatment.

Alternatively stated, Jakobovits discloses a completely artificial ligand-receptor binding system. Contrary to the Examiner's assertion, the use of such an artificial system is completely excluded from the scope of the current claims. *See, e.g.*, Claim 1 ("which cells or organelles have not been surface-treated"). The use of "comprising" language does not undo the effects of the specific claim language chosen by Applicant. Thus, without more, Jakobovits is limited to the lectin-receptor interactions on enzymatically-treated cells disclosed, a system outside the scope of the claimed methods.

Edelman fails to cure this deficiency in Jakobovits. As was the case in Jakobovits, the Examiner appears to be relying solely on Edelman's introductory statements regarding a general need for means to isolate cell surface markers to extend what is otherwise disclosure related to a completely different methodology. Edelman is unambiguously and exclusively directed to the isolation of purified cell populations. In fact, beyond the single sentence in the introductory paragraph, Edelman lacks any specific mention of methods useful in the isolation of receptors, much less receptors associated with additional non-covalently associated cellular components. Applicant does not believe that this is an arbitrary interpretation of Edelman, but rather a reasoned reading of the complete reference. For example, the title of the article concerns the fractionation of cells, not cell receptors. Edelman goes on to describe the main requirements for the methods as issue - manipulations of cell populations. *See* Edelman at 195, first ¶. Edelman goes on to describe the methods as facilitating characterization of cells having at least one property. *See* Edelman at 195, second ¶. Simply stated, Edelman describes affinity and fiber fractionation of cells without any suggestion or teaching regarding the isolation of receptors with their associated microenvironment. *See* Edelman at 197-98. Because Edelman fails to teach any isolation of receptors with their associated microenvironments, the ordinary artisan would find no motivation or suggestion to combine the teachings of Jakobovits with Edelman.

Applicant submits that Edelman actually *teaches away* from the removal of the cell receptors from the cell surface. When Edelman describes the removal of the cells from the fibers used, absolutely no mention is made of the isolation of the receptors that may bind the ligands on the fibers. *See* Edelman at page 208-10. Thus, while Figure 1 appears to suggest that mechanical fiber fractionation results in the ligand remaining attached to the receptor, Edelman provides no objective evidence that this is the actual result of the separation process employed. In fact, the uses that Edelman describes for the fractionated cells require that the receptors on the fractionated cell population are intact, suggesting that Edelman's method does not result in the removal of the receptors from the cell surface. For example, Edelman teaches that cells can be immediately retested for binding to the selection ligand after fractionation. *See* Edelman at page 214, first full ¶. In this example, Edelman discloses that there is a first fractionation mediated by a "plucking" of the fibers binding the cells. First, Applicant again notes that it is the fiber that is "plucked" in Edelman,

plucking as one would pluck a string while playing a guitar. This is *distinct* from the "plucking" of the instant methods where a receptor and its associated microenvironment are plucked from the cell membrane using a dissociating force, plucking as one would pluck a peach blossom from its tree. Second, in Edelman's example, the solution containing the cells is then immediately added to a second fractionation with the same ligand used to fractionate the cells in order to determine the specificity obtained in the original fraction. Such an assay design cannot predict specificity if the receptor has been removed from the cell surface because the receptor would be absent or significantly diminished by its removal. This is particularly relevant for the specific ligand-receptor interaction that has limited numbers of receptors on the cell surface - a limitation typically not associated with lectin-receptor binding. In other words, if the teachings of Jakobovits were modified to isolate non-lectin cell receptors from the cell surface, Edelman's method would be unsatisfactory for its stated intended purpose - to purify a discrete population of cells with fully functional receptors. Therefore, there is no suggestion or motivation to make such a combination. *See* MPEP § 2143.01 ("If proposed modification would render the prior art invention being modified unsatisfactory for its intended purpose, then there is no suggestion or motivation to make the proposed modification.").

Applicant continues to maintain that the combination of Jakobovits and Edelman fails to provide enabling disclosure for the instant methods. First and foremost, enabling disclosure for the isolation of the receptor/ligand complexes is absent from the cited combination of references. The Examiner points to a 27 kD band allegedly corresponding to PNA, *i.e.*, the lectin used as solid phase ligand, on pages 1486-87 as evidence that Jakobovits teaches isolation of the receptor/ligand complexes. However, Jakobovits does not indicate definitively that this is PNA. More specifically, Jakobovits describes the band at 27 kD as "probably corresponding to the PNA subunit" at page 1486 and "suggests the presence of a membrane protein comigrating on the gels with the PNA subunit" at page 1487. It is difficult to believe that the ordinary artisan would consider the description of what is clearly considered an artifact of the elution process a teaching for the deliberate and complete recovery of the solid support bound ligand. The fact that contaminating PNA subunits may have been present after elution gives no indication as to why and how the ligand should be removed from the solid support. Edelman is completely silent with regards to the

isolation of the ligand off of the solid support. Thus, the combination fails to teach how to make or use methods that require the isolation of both the ligand-bound to the solid support as well as its cell surface receptor and associated microenvironment. In the absence of such teachings, Jakobovits and the Edelman references fail to render the instant methods *prima facie* obvious.

Applicant still maintains that the isolation of lection receptors from non-nucleated cells, *i.e.*, erythrocytes, fails to enable methods for isolation of non-lectin receptors using nucleated cells. Jakobovits itself supports this assertion in its statement that the authors were investigating the usefulness of the disclosed lectin-ligand system in nucleated cells in subsequent experiments. Applicant notes that while many membrane properties are commonly shared between various cell types, erythrocytes are distinct from nucleated cells in a number of ways that likely impact the success of receptor isolation methods. For example, erythrocytes have a single outer membrane that is in great excess relative to that of nucleated cells and is easily deformed. *See* Exhibit A at page 382. Erythrocyte membranes also have considerably higher cholesterol content than most other nucleated cells, which greatly influences membrane fluidity, and thereby enhances the mobility of receptors in the membrane. *See* Exhibit B at 333-34. This characteristic likely synergizes with the numerous lectin receptors available on the surface of a neuraminidase-treated erythrocyte to enhance binding and subsequent removal of the receptors from the membrane because the receptors are very mobile within the membrane and therefore even more are available for binding that in a nucleated cell with a less fluid membrane. Thus, it is not immediately apparent that the methodology used for erythrocytes is adequate or even relevant for non-lectin receptor isolation in nucleated cells because of at least these differences in receptor number and membrane dynamics. Indeed, even Jakobovits acknowledges that such teachings are not immediately extendible to nucleated cells without further experimentation being performed.

Applicant submits that the claimed isolation of a receptor and its associated microenvironment is distinct from the teachings of Edelman. First, while Applicant acknowledges that Edelman discloses the possibility of a lesion in the membrane resulting from the cell fractionation method taught, it is merely hypothesized and not demonstrated in either Edelman reference. *See* Edelman at page 208 (stating that "[t]his shearing of the cell-fiber bond may produce a lesion in the cell surface membrane") (emphasis added) and Edelman at page 209 (hypothesizing

that viability increases with incubation serum, "suggesting that the cells are repairing lesions in their surface membranes") (emphasis added). Such language without more does not prove that a lesion in the cell surface membrane existed. Moreover, there is no objective evidence in Edelman to support the Examiner's assertion that a lesion implies a tear in the membrane due to the association of some of the membrane components with the receptor on the solid phase-bound ligand. Edelman provides no objective evidence of additional membrane components or even the receptor itself being associated with the solid support-bound ligand. The hypothetical interaction disclosed in Figure 5 of the '324 patent does not show the retention of any membranous component in addition to the receptor. Furthermore, given the stated purpose of Edelman, *i.e.*, to isolate purified populations of fully functional cells, the removal of a significant number of cell surface receptors and associated cellular components would be undesirable and contrary to Edelman's purpose. It is well known in the art that such a lesion would not be repaired in the 30 minute incubation time disclosed by Edelman and likely would seriously comprise the functionality of the cell. Finally, Applicant amends herein the term "microenvironment" to further clarify that the claimed method results in the isolation of the receptor and its microenvironment including one or more non-covalently associated cellular components. The combination of Jakobovits and Edelman fails to teach a method of isolation for a receptor and its associated microenvironment, and therefore fails to render the instant methods *prima facie* obvious.

Finally, the additional cited references of Chang, Kupchik, and Seifert fail to cure the deficiencies in Jakobovits and the Edelman references. Thus, these additional references fail to render the instant methods *prima facie* obvious.

In view of the above, Applicant respectfully submits that the basis of the rejection may be withdrawn.

Rejection Under the Judicially Created Doctrine of Obviousness-Type Double Patenting

Claims 1, 4-18, 20, 22, 24, and 27-31 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-4, 16-25, and 28-31 of co-pending Application Serial No. 10/209,328. According to the Examiner, the

conflicting claims are not identical, but they are not patentably distinct from each other because both sets of claims encompass common subject matter. Applicant traverses this rejection.

As no allowable subject matter has been indicated in either application, Applicant requests that this rejection be held in abeyance until such time. Applicant will submit a terminal disclaimer once allowable subject matter is indicated in the present application if it is required.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 511582006000. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: April 22, 2004

Respectfully submitted,

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Red Blood Cells, Anemia, and Polycythemia

With this chapter we begin discussing the *blood cells* and cells of the *macrophage system* and *lymphatic system.* We first present the functions of red blood cells, which are the most abundant of all the cells of the blood and are necessary for delivery of oxygen to the tissues.

RED BLOOD CELLS (ERYTHROCYTES)

The major function of red blood cells, also known as *erythrocytes,* is to transport *hemoglobin,* which in turn carries oxygen from the lungs to the tissues. In some lower animals, hemoglobin circulates as free protein in the plasma, not enclosed in red blood cells. When it is free in the plasma of the human being, about 3 per cent of it leaks through the capillary membrane into the tissue spaces or through the glomerular membrane of the kidney into the glomerular filtrate each time the blood passes through the capillaries. Therefore, for hemoglobin to remain in the blood stream, it must exist inside red blood cells.

The red blood cells have other functions besides transport of hemoglobin. For instance, they contain a large quantity of *carbonic anhydrase,* which catalyzes the reversible reaction between carbon dioxide and water, increasing the rate of this reaction several thousandfold. The rapidity of this reaction makes it possible for the water of the blood to transport enormous quantities of carbon dioxide from the tissues to the lungs in the form of the bicarbonate ion (HCO_3^-). Also, the hemoglobin in the cells is an excellent *acid-base buffer* (as is true of most proteins), so that the red blood cells are responsible for most of the acid-base buffering power of whole blood.

Shape and Size of Red Blood Cells. Normal red blood cells, shown in Figure 32–3, are biconcave discs having a mean diameter of about 7.8 micrometers and a thickness at the thickest point of 2.5 micrometers and in the center of 1 micrometer or less. The average volume of the red blood cell is 90 to 95 cubic micrometers.

The shapes of red blood cells can change remarkably as the cells squeeze through capillaries. Actually, the red blood cell is a "bag" that can be deformed into almost any shape. Furthermore, because the normal cell has a great excess of cell membrane for the quantity of material inside, deformation does not stretch the membrane greatly and, consequently, does not rupture the cell, as would be the case with many other cells.

Concentration of Red Blood Cells in the Blood. In normal men, the average number of red blood cells per cubic millimeter is 5,200,000 (± 300,000) and in normal women, 4,700,000 (± 300,000). Persons living at high altitudes have greater numbers of red blood cells. This is discussed later.

Quantity of Hemoglobin in the Cells. Red blood cells have the ability to concentrate hemoglobin in the cell fluid up to about 34 g/dl of cells. The concentration does not rise above this value because this is a metabolic limit of the cell's hemoglobin-forming mechanism. Furthermore, in normal people, the percentage of hemoglobin is almost always near the maximum in each cell. However, when hemoglobin formation is deficient, the percentage of hemoglobin in the cells may fall considerably below this value, and the volume of the red cell may also decrease because of diminished hemoglobin to fill the cell.

When the hematocrit (the percentage of the blood that is cells—normally 40 to 45 per cent) and the quantity of hemoglobin in each respective cell are normal, the whole blood of men contains an average of 16 grams of hemoglobin per deciliter and of women, an average of 14 g/dl. As discussed in connection with blood transport of oxygen in Chapter 40, each gram of pure hemoglobin is capable of combining with 1.39 milliliters of oxygen. Therefore, in a normal man, more than 21 milliliters of oxygen can be carried in combination with hemoglobin in each deciliter of blood, and in a normal woman, 19 milliliters of oxygen can be carried.

Production of Red Blood Cells

Areas of the Body That Produce Red Blood Cells. In the early few weeks of embryonic life, primitive, nucleated red blood cells are produced in the *yolk sac.* During the middle trimester of gestation, the *liver* is the main organ for production of red blood cells, although reasonable numbers of red blood cells are also produced in the *spleen* and *lymph nodes.* Then, during the last month or so of gestation and after birth, red blood cells are produced exclusively in the *bone marrow.*

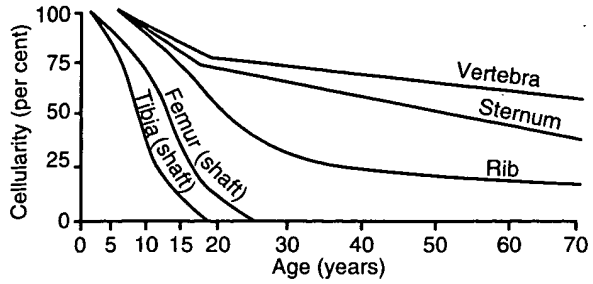

FIGURE 32-1

Relative rates of red blood cell production in the bone marrow of different bones at different ages.

As demonstrated in Figure 32–1, the bone marrow of essentially all bones produces red blood cells until a person is 5 years old; but the marrow of the long bones, except for the proximal portions of the humeri and tibiae, becomes quite fatty and produces no more red blood cells after about age 20 years. Beyond this age, most red cells are produced in the marrow of the membranous bones, such as the vertebrae, sternum, ribs, and ilia. Even in these bones, the marrow becomes less productive as age increases.

Genesis of Blood Cells

Pluripotential Hematopoietic Stem Cells, Growth Inducers, and Differentiation Inducers. In the red cell–producing bone marrow are cells called *pluripotential hematopoietic stem cells,* from which all the cells in the circulating blood are derived. Figure 32–2 shows the successive divisions of the pluripotential cells to form the different peripheral blood cells. As these cells reproduce, continuing throughout life, a small portion of them remains exactly like the original pluripotential cells and is retained in the bone marrow to maintain a supply of these, although their numbers do diminish with age. Most of the reproduced stem cells, however, differentiate to form the other cells shown to the right in Figure 32–2. The early offspring cells still cannot be recognized as different from the pluripotential stem cells, even though they have already become committed to a particular line of cells and are called *committed stem cells.*

The different committed stem cells, when grown in culture, will produce colonies of specific types of blood cells. A committed stem cell that produces erythrocytes is called a *colony-forming unit–erythrocyte,* and the abbreviation CFU-E is used to designate this type of stem cell. Likewise, colony-forming units that form granulocytes and monocytes have the designation CFU-GM, and so forth.

Growth and reproduction of the different stem cells are controlled by multiple proteins called *growth inducers.* Four major growth inducers have been described, each having different characteristics. One of these, *interleukin-3,* promotes growth and reproduction of virtually all the different types of stem cells, whereas the others induce growth of only specific types of committed stem cells.

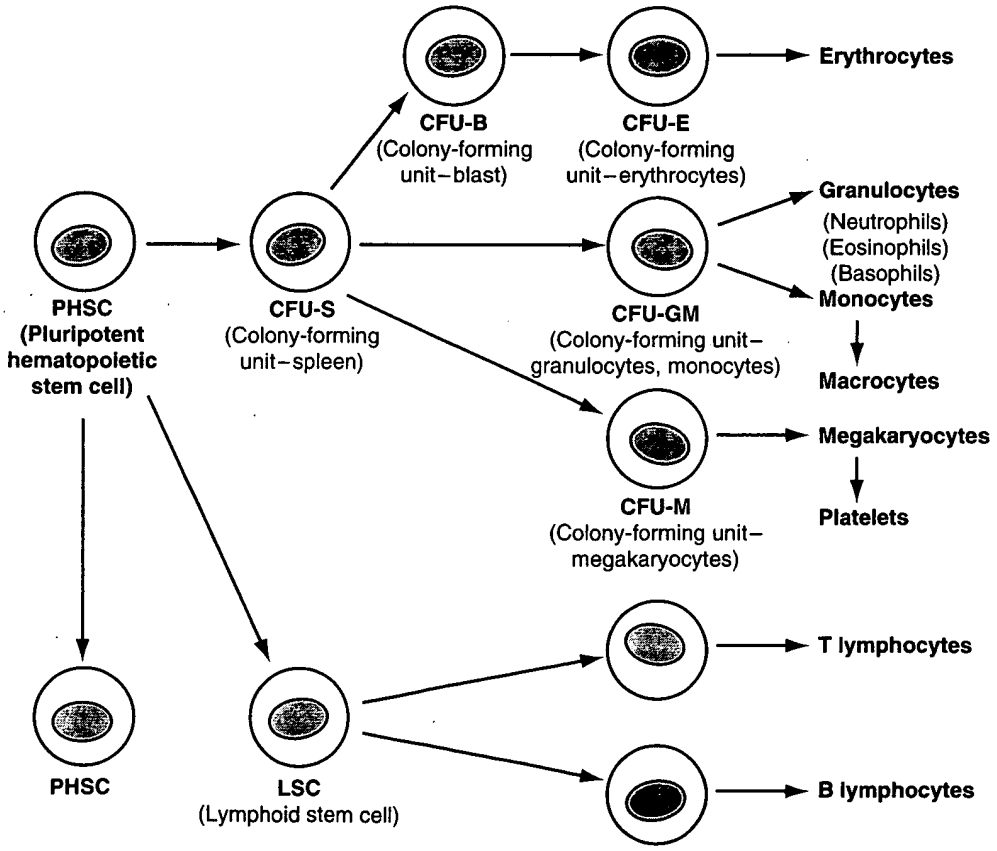

FIGURE 32-2

Formation of the multiple different peripheral blood cells from the original pluripotential hematopoietic stem cell (PHSC) in the bone marrow.

The growth inducers promote growth but not differentiation of the cells. This is the function of still another set of proteins called *differentiation inducers.* Each of these causes one type of stem cell to differentiate one or more steps toward a final type of adult blood cell.

Formation of the growth inducers and differentiation inducers is itself controlled by factors outside the bone marrow. For instance, in the case of red blood cells, exposure of the body to low oxygen for a long time results in growth induction, differentiation, and production of greatly increased numbers of erythrocytes, as we discuss later in the chapter. In the case of some of the white blood cells, infectious diseases cause growth, differentiation, and eventual formation of specific types of white blood cells that are needed to combat the infection.

Stages of Differentiation of Red Blood Cells

The first cell that can be identified as belonging to the red blood cell series is the *proerythroblast,* shown in Figure 32–3. Under appropriate stimulation, large numbers of these cells are formed from the CFU-E stem cells.

Once the proerythroblast has been formed, it divides multiple times, eventually forming many mature red blood cells. The first-generation cells are called *basophil erythroblasts* because they stain with basic dyes; the cell at this time has accumulated very little hemoglobin. In the succeeding generations, as shown in Figure 32–3, the cells become filled with hemoglobin to a concentration of about 34 per cent, the nucleus condenses to a small size, and its final remnant is extruded from the cell. At the same time, the endoplasmic reticulum is reabsorbed. The cell at this stage is called a *reticulocyte* because it still contains a small amount of basophilic material, consisting of remnants of the Golgi apparatus, mitochondria, and a few other cytoplasmic organelles. During this reticulocyte stage, the cells pass from the bone marrow into the blood capillaries by diapedesis (squeezing through the pores of the capillary membrane).

The remaining basophilic material in the reticulocyte normally disappears within 1 to 2 days, and the cell is then the *mature erythrocyte.* Because of the short life of the reticulocytes, their concentration among all the red cells of the blood is normally slightly less than 1 per cent.

Regulation of Red Blood Cell Production—Role of Erythropoietin

The total mass of red blood cells in the circulatory system is regulated within narrow limits, so that an adequate number of red cells is always available to provide sufficient transport of oxygen from the lungs to the tissues, yet the cells do not become so numerous that they impede blood flow. What we know about this

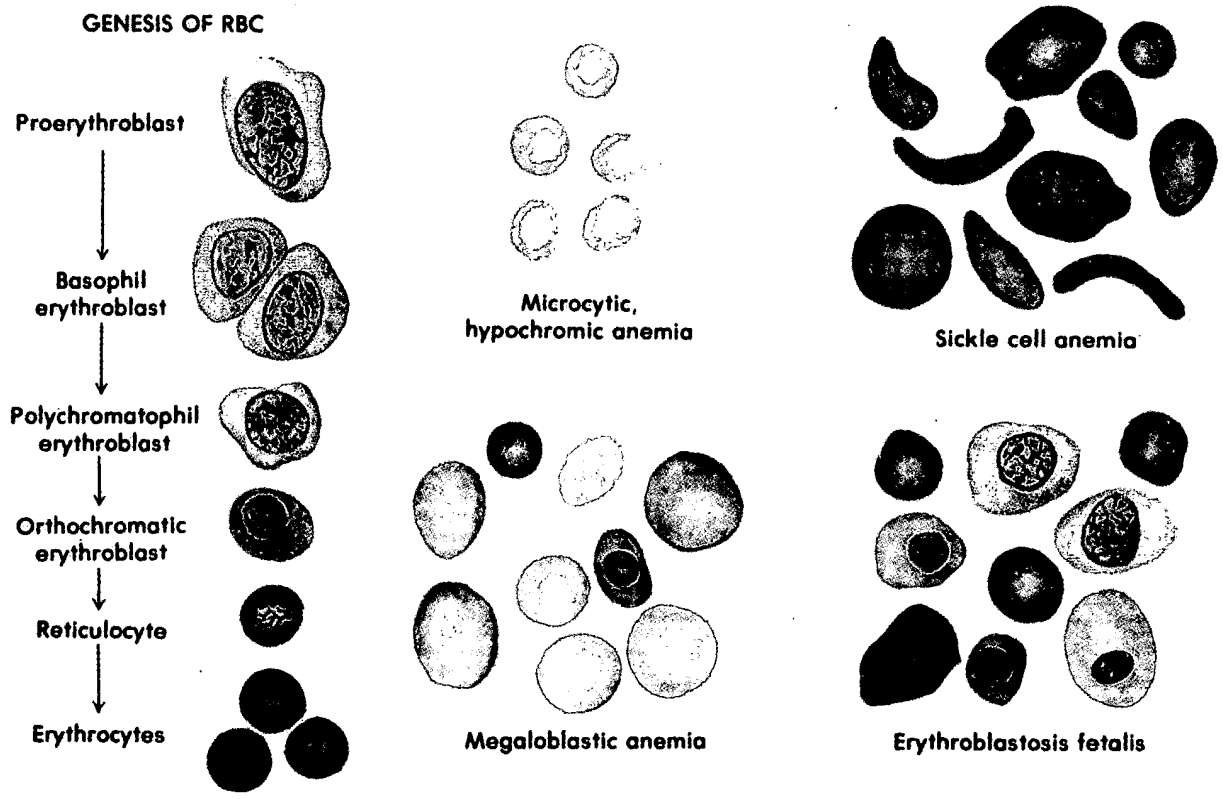

FIGURE 32–3

Genesis of normal red blood cells, and red blood cells in different types of anemias.

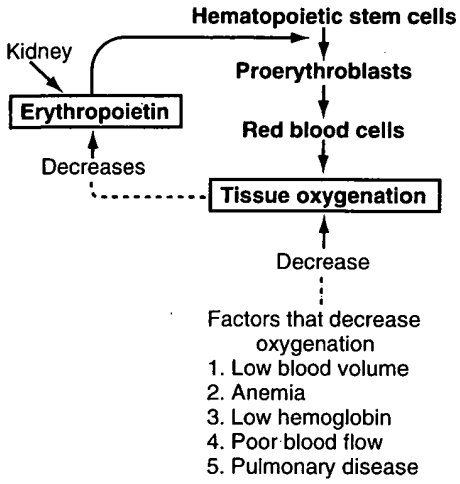

FIGURE 32-4

Function of the erythropoietin mechanism to increase production of red blood cells when various factors decrease tissue oxygenation.

control mechanism is diagrammed in Figure 32–4 and is as follows.

Tissue Oxygenation as the Most Essential Regulator of Red Blood Cell Production. Any condition that causes the quantity of oxygen transported to the tissues to decrease ordinarily increases the rate of red blood cell production. Thus, when a person becomes extremely *anemic* as a result of hemorrhage or any other condition, the bone marrow immediately begins to produce large quantities of red blood cells. Also, destruction of major portions of the bone marrow by any means, especially by x-ray therapy, causes hyperplasia of the remaining bone marrow, thereby attempting to supply the demand for red blood cells in the body.

At very *high altitudes,* where the quantity of oxygen in the air is greatly decreased, insufficient oxygen is transported to the tissues, and red cell production is greatly increased. It is not the concentration of red blood cells in the blood that controls the rate of red cell production but the functional ability of the red cells to transport oxygen to the tissues in relation to the tissue demand for oxygen.

Various diseases of the circulation that cause decreased blood flow through the peripheral vessels, and particularly those that cause failure of oxygen absorption by the blood as it passes through the lungs, can also increase the rate of red cell production. This is especially apparent in prolonged *cardiac failure* and in many *lung diseases* because the tissue hypoxia resulting from these conditions increases the rate of red cell production, with a resultant increase in the hematocrit and usually in the total blood volume as well.

Erythropoietin, Its Function to Stimulate Red Cell Production, and Its Formation in Response to Hypoxia. The principal factor that stimulates red blood cell production in low oxygen states is a circulating hormone called *erythropoietin,* a glycoprotein with a molecular weight of about 34,000. In the absence of erythropoietin, hypoxia has little or no effect in stimulating red blood cell production. Conversely, when the erythropoietin system is functional, hypoxia causes marked increase in erythropoietin production, and the erythropoietin in turn enhances red blood cell production until the hypoxia is relieved.

Role of the Kidneys in Formation of Erythropoietin. In the normal person, about 90 per cent of all erythropoietin is formed in the kidneys; the remainder is formed mainly in the liver. It is not known exactly where in the kidneys the erythropoietin is formed. One likely possibility is that the renal tubular epithelial cells secrete the erythropoietin because anemic blood is unable to deliver enough oxygen from the peritubular capillaries to the highly oxygen-consuming tubular cells, thus stimulating erythropoietin production.

At times, hypoxia in other parts of the body but not in the kidneys will also stimulate kidney erythropoietin secretion, which suggests that there might be some nonrenal sensor that sends an additional signal to the kidneys to produce this hormone. In particular, both norepinephrine and epinephrine and several of the prostaglandins stimulate erythropoietin production.

When both kidneys are removed from a person or when the kidneys are destroyed by renal disease, the person invariably becomes very anemic because the 10 per cent of the normal erythropoietin formed in other tissues (mainly in the liver) is sufficient to cause only one third to one half as much red blood cell formation as needed by the body.

Effect of Erythropoietin in Erythrogenesis. When an animal or a person is placed in an atmosphere of low oxygen, erythropoietin begins to be formed within minutes to hours, and it reaches maximum production within 24 hours. Yet almost no new red blood cells appear in the circulating blood until about 5 days later. From this fact, as well as still other studies, it has been determined that the important effect of erythropoietin is to stimulate the production of proerythroblasts from hemopoietic stem cells in the bone marrow. In addition, once the proerythroblasts are formed, the erythropoietin causes these cells also to pass more rapidly through the different erythroblastic stages than they normally do, further speeding up the production of new cells. The rapid production of cells continues as long as the person remains in the low oxygen state or until enough red blood cells have been produced to carry adequate amounts of oxygen to the tissues despite the low oxygen; at this time, the rate of erythropoietin production decreases to a level that will maintain the required number of red cells but not an excess.

In the absence of erythropoietin, few red blood cells are formed by the bone marrow. At the other extreme, when large quantities of erythropoietin are formed and if there is plenty of iron available and other required nutrients, the rate of red blood cell production can rise to perhaps 10 or more times normal. Therefore, the

erythropoietin control mechanism for red blood cell production is a powerful one.

Maturation of Red Blood Cells—Requirement for Vitamin B_{12} (Cyanocobalamin) and Folic Acid

Because of the continuing need to replenish red blood cells, the erythropoietic cells of the bone marrow are among the most rapidly growing and reproducing cells of the entire body. Therefore, as would be expected, their maturation and rate of production are affected greatly by a person's nutritional status.

Especially important for final maturation of the red blood cells are two vitamins, *vitamin B_{12}* and *folic acid.* Both of these are essential for the synthesis of DNA because each in a different way is required for the formation of thymidine triphosphate, one of the essential building blocks of DNA. Therefore, lack of either vitamin B_{12} or folic acid causes diminished DNA and, consequently, failure of nuclear maturation and division. Furthermore, the erythroblastic cells of the bone marrow, in addition to failing to proliferate rapidly, produce mainly larger than normal red cells called *macrocytes,* and the cell has a flimsy membrane and is often irregular, large, and oval instead of the usual biconcave disc. These poorly formed cells, after entering the circulating blood, are capable of carrying oxygen normally, but their fragility causes them to have a short life, one-half to one-third normal. Therefore, it is said that either vitamin B_{12} or folic acid deficiency causes *maturation failure* in the process of erythropoiesis.

The cause of the abnormal cells seems to be as follows: The inability of the cells to synthesize adequate quantities of DNA leads to slow reproduction of the cells. Because of abnormalities of the DNA, the structural components of the cell membrane and cytoskeleton are also malformed, which leads to the abnormal cell shapes and especially the greatly increased cell membrane fragility.

Maturation Failure Caused by Poor Absorption of Vitamin B_{12}—Pernicious Anemia. A common cause of maturation failure is failure to absorb vitamin B_{12} from the gastrointestinal tract. This often occurs in the disease *pernicious anemia,* in which the basic abnormality is an *atrophic gastric mucosa* that fails to secrete normal gastric secretions. The parietal cells of the gastric glands secrete a glycoprotein called *intrinsic factor,* which combines with vitamin B_{12} in food and makes the B_{12} available for absorption by the gut. It does this in the following way: (1) The intrinsic factor binds tightly with the vitamin B_{12}. In this bound state, the B_{12} is protected from digestion by the gastrointestinal enzymes. (2) Still in the bound state, the intrinsic factor binds to specific receptor sites on the brush border membranes of the mucosal cells in the ileum. (3) Vitamin B_{12} is transported into the blood during the next few hours by the process of pinocytosis, carrying the intrinsic factor and the vitamin together through the membrane.

Lack of intrinsic factor, therefore, causes loss of much of the vitamin because of both digestive enzyme action in the gut and failure of its absorption.

Once vitamin B_{12} has been absorbed from the gastrointestinal tract, it is stored in large quantities in the liver and then released slowly as needed to the bone marrow and other tissues of the body. The minimum amount of vitamin B_{12} required each day to maintain normal red cell maturation is only 1 to 3 micrograms, and the normal store in the liver and other body tissues is about 1000 times this amount. Therefore, 3 to 4 years of defective B_{12} absorption are required to cause maturation failure anemia.

Failure of Maturation Caused by Deficiency of Folic Acid (Pteroylglutamic Acid). Folic acid is a normal constituent of green vegetables, some fruits, liver, and other meats. However, it is easily destroyed during cooking. Also, people with gastrointestinal absorption abnormalities, such as the frequently occurring small intestinal disease called *sprue,* often have serious difficulty in absorbing both folic acid and vitamin B_{12}. Therefore, in many instances of maturation failure, the cause is deficiency of intestinal absorption of both folic acid and vitamin B_{12}.

Formation of Hemoglobin

Synthesis of hemoglobin begins in the proerythroblasts and continues even into the reticulocyte stage because when the reticulocytes leave the bone marrow and pass into the blood stream, they continue to form minute quantities of hemoglobin for another day or so.

Figure 32–5 shows the basic chemical steps in the formation of hemoglobin. First, succinyl-CoA, formed in the Krebs cycle as explained in Chapter 67, binds with glycine to form a pyrrole molecule. In turn, four pyrroles combine to form protoporphyrin IX, which then combines with iron to form the *heme* molecule. Finally, each heme molecule combines with a long polypeptide chain, called a *globin,* synthesized by the ribosomes, forming a subunit of hemoglobin called a *hemoglobin chain* (Figure 32–6). Each of these chains has a molecular weight of about 16,000; four of them in turn bind together loosely to form the whole hemoglobin molecule.

There are several slight variations in different subunit hemoglobin chains, depending on the amino acid composition of the polypeptide portion. The different types

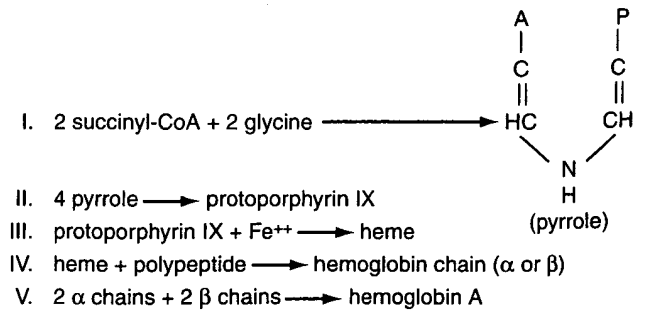

FIGURE 32–5

Formation of hemoglobin.

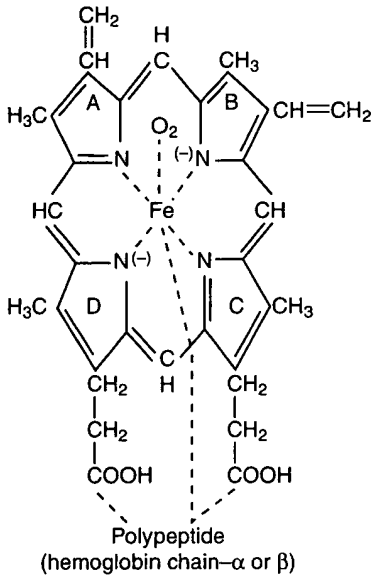

FIGURE 32-6

Basic structure of the hemoglobin molecule, showing one of the four heme chains that bind together to form the hemoglobin molecule.

of chains are designated *alpha chains, beta chains, gamma chains,* and *delta chains.* The most common form of hemoglobin in the adult human being, *hemoglobin A,* is a combination of *two alpha chains* and *two beta chains.* Hemoglobin A has a molecular weight of 64,458.

Because each hemoglobin chain has a heme prosthetic group containing an atom of iron, and because there are four hemoglobin chains in each hemoglobin molecule, one finds four iron atoms in each hemoglobin molecule; each of these can bind with one molecule of oxygen, making a total of four molecules of oxygen (or eight oxygen atoms) that can be transported by each hemoglobin molecule.

The nature of the hemoglobin chains determines the binding affinity of the hemoglobin for oxygen. Abnormalities of the chains can alter the physical characteristics of the hemoglobin molecule as well. For instance, in *sickle cell anemia,* the amino acid *valine* is substituted for *glutamic acid* at one point in each of the two beta chains. When this type of hemoglobin is exposed to low oxygen, it forms elongated crystals inside the red blood cells that are sometimes 15 micrometers in length. These make it almost impossible for the cells to pass through many small capillaries, and the spiked ends of the crystals are likely to rupture the cell membranes, thus leading to sickle cell anemia.

Combination of Hemoglobin with Oxygen. The most important feature of the hemoglobin molecule is its ability to combine loosely and reversibly with oxygen. This ability is discussed in detail in Chapter 40 in relation to respiration because the primary function of hemoglobin in the body is its ability to combine with oxygen in the lungs and then to release this oxygen readily in the tissue capillaries where the gaseous tension of oxygen is much lower than in the lungs.

Oxygen *does not* combine with the two positive bonds of the iron in the hemoglobin molecule. Instead, it binds loosely with one of the so-called coordination bonds of the iron atom. This is an extremely loose bond so that the combination is easily reversible. Furthermore, the oxygen does not become ionic oxygen but is carried as molecular oxygen, composed of two oxygen atoms, to the tissues, where, because of the loose, readily reversible combination, it is released into the tissue fluids still in the form of molecular oxygen, rather than ionic oxygen.

Iron Metabolism

Because iron is important for the formation of hemoglobin, myoglobin, and other substances such as the cytochromes, cytochrome oxidase, peroxidase, and catalase, it is essential to understand the means by which iron is utilized in the body.

The total quantity of iron in the body averages 4 to 5 grams, about 65 per cent of which is in the form of hemoglobin. About 4 per cent is in the form of myoglobin, 1 per cent is in the form of the various heme compounds that promote intracellular oxidation, 0.1 per cent is combined with the protein transferrin in the blood plasma, and 15 to 30 per cent is stored mainly in the reticuloendothelial system and liver parenchymal cells, principally in the form of ferritin.

Transport and Storage of Iron. Transport, storage, and metabolism of iron in the body are diagrammed in Figure 32–7 and may be explained as follows: When iron is absorbed from the small intestine, it immediately combines in the blood plasma with a beta globulin, *apotransferrin,* to form *transferrin,* which is then transported in the plasma. The iron is loosely bound in the transferrin and, consequently, can be released to any of the tissue cells at any point in the body. Excess iron in the blood is deposited in all cells of the body, but *especially* in the liver hepatocytes and less in the reticuloendothelial cells of the bone marrow. In the receiving cell cytoplasm, the iron combines mainly with a protein, *apoferritin,* to form *ferritin.* Apoferritin has a molecular weight of about 460,000, and varying quantities of iron can combine in clusters of iron radicals with this large molecule; therefore, ferritin may contain only a small amount of iron or a large amount. This iron stored as ferritin is called *storage iron.*

Smaller quantities of the iron in the storage pool are stored in an extremely insoluble form called *hemosiderin.* This is especially true when the total quantity of iron in the body is more than the apoferritin storage pool can accommodate. Hemosiderin forms especially large clusters in the cells and, consequently, can be stained and observed microscopically as large particles in tissue slices. Ferritin can also be stained, but the ferritin particles are so small and dispersed that they usually can be seen only with the electron microscope.

When the quantity of iron in the plasma falls very low, iron is removed from ferritin quite easily but from

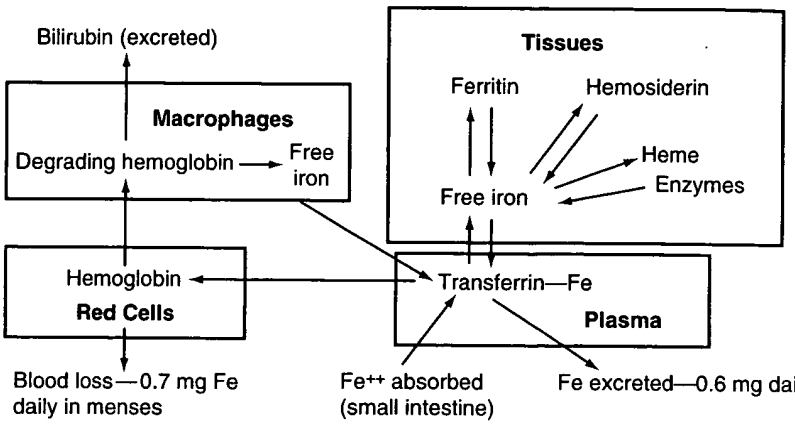

FIGURE 32-7
Iron transport and metabolism.

hemosiderin much less easily. The iron is then transported again in the form of transferrin in the plasma to the portions of the body where it is needed.

A unique characteristic of the transferrin molecule is that it binds strongly with receptors in the cell membranes of erythroblasts in the bone marrow. Then, along with its bound iron, it is ingested into the erythroblasts by endocytosis. There the transferrin delivers the iron directly to the mitochondria, where heme is synthesized. In people who do not have adequate quantities of transferrin in their blood, failure to transport iron to the erythroblasts in this manner can cause severe hypochromic anemia—that is, red cells that contain much less hemoglobin than normal.

When red blood cells have lived their life span and are destroyed, the hemoglobin released from the cells is ingested by the cells of the monocyte-macrophage system. There free iron is liberated, and it is then mainly stored in the ferritin pool or reused for formation of new hemoglobin.

Daily Loss of Iron. A man excretes about 0.6 milligram of iron each day, mainly into the feces. Additional quantities of iron are lost whenever bleeding occurs. For a woman, the menstrual loss of blood brings the iron loss to an average value of about 1.3 mg/day.

Absorption of Iron from the Gastrointestinal Tract

Iron is absorbed from all parts of the small intestine, mostly by the following mechanism. The liver secretes moderate amounts of *apotransferrin* into the bile that flows through the bile duct into the duodenum. In the small intestine, the apotransferrin binds with free iron and also with certain iron compounds such as hemoglobin and myoglobin from meat, two of the most important sources of iron in the diet. This combination is called *transferrin.* It in turn is attracted to and binds with receptors in the membranes of the intestinal epithelial cells. Then, by pinocytosis, the transferrin molecule, carrying with it its iron store, is absorbed into the epithelial cells and later is released into the blood capillaries beneath these cells in the form of *plasma transferrin.*

The rate of iron absorption is extremely slow, with a maximum rate of only a few milligrams per day. This means that when tremendous quantities of iron are present in the food, only small proportions of this can be absorbed.

Regulation of Total Body Iron by Controlling Rate of Absorption. When the body has become saturated with iron so that essentially all the apoferritin in the iron storage areas is already combined with iron, the rate of absorption of iron from the intestinal tract becomes greatly decreased. Conversely, when the iron stores have been depleted of iron, the rate of absorption can become accelerated probably to five or more times as great as when the iron stores are normally saturated. Thus, the total body iron is regulated to a great extent by altering the rate of absorption.

Feedback Mechanisms for Regulating Iron Absorption. Two mechanisms that play at least some role in regulating iron absorption are the following: (1) When essentially all the apoferritin in the body has become saturated with iron, it becomes difficult for transferrin to release iron to the tissues. As a consequence, the transferrin, which is normally only one-third saturated with iron, now becomes almost fully bound with iron, so that the transferrin accepts almost no new iron from the mucosal cells of the intestines. Then, as a final stage of this process, the buildup of excess iron in the mucosal cells themselves depresses active absorption of iron from the intestinal lumen. (2) When the body already has excess stores of iron, the liver decreases its rate of formation of apotransferrin, thus reducing the concentration of this iron-transporting molecule in the plasma and the bile. Therefore, less iron is absorbed by the intestinal apotransferrin mechanism, and less iron can be transported away from the intestinal epithelial cells in the plasma by plasma transferrin.

Yet, despite these feedback control mechanisms for regulating iron absorption, when a person eats extremely large amounts of iron compounds, excess iron does enter the blood and can lead to massive deposition of hemosiderin in the reticuloendothelial cells throughout the body. At times, this can be very damaging.

DESTRUCTION OF RED BLOOD CELLS

When red blood cells are delivered from the bone marrow into the circulatory system, they normally circulate an average of 120 days before being destroyed. Even though mature red cells do not have a nucleus, mitochondria, or endoplasmic reticulum, they do have cytoplasmic enzymes that are capable of metabolizing glucose and forming small amounts of adenosine triphosphate. These enzymes also (1) maintain the pliability of the cell membrane, (2) maintain membrane transport of ions, (3) keep the iron of the cells' hemoglobin in the ferrous form rather than the ferric form (which causes the formation of methemoglobin, which will not carry oxygen), and (4) prevent oxidation of the proteins in the red cells. Even so, with aging, the metabolic systems of the red cells become progressively less active, and the cells become more and more fragile, presumably because their life processes wear out.

Once the red cell membrane becomes fragile, the cell ruptures during passage through some tight spot of the circulation. Many of the red cells self-destruct in the spleen, where they squeeze through the red pulp of the spleen. Here the spaces between the structural trabeculae of the red pulp, through which most of the cells must pass, are only 3 micrometers wide, in comparison with the 8-micrometer diameter of the red cell. When the spleen is removed, the number of abnormal red cells and old cells circulating in the blood increases considerably.

Destruction of Hemoglobin. When red blood cells burst and release their hemoglobin, the hemoglobin is phagocytized almost immediately by macrophages in many parts of the body, but especially by the Kupffer cells of the liver and macrophages of the spleen and bone marrow. During the next few hours to days, the macrophages release the iron from the hemoglobin and pass it back into the blood to be carried by transferrin either to the bone marrow for production of new red blood cells or to the liver and other tissues for storage in the form of ferritin. The porphyrin portion of the hemoglobin molecule is converted by the macrophages, through a series of stages, into the bile pigment *bilirubin,* which is released into the blood and later secreted by the liver into the bile; this is discussed in relation to liver function in Chapter 70.

THE ANEMIAS

Anemia means a deficiency of hemoglobin, which can be caused by either too few red blood cells or too little hemoglobin in the cells. Some types of anemia and their physiologic causes are the following.

Blood Loss Anemia. After rapid hemorrhage, the body replaces the fluid portion of the plasma in 1 to 3 days, but this leaves a low concentration of red blood cells. If a second hemorrhage does not occur, the red blood cell concentration usually returns to normal within 3 to 6 weeks.

In chronic blood loss, a person frequently cannot absorb enough iron from the intestines to form hemoglobin as rapidly as it is lost. Red cells are then produced that are much smaller than normal and have too little hemoglobin inside them, giving rise to *microcytic, hypochromic anemia,* which is shown in Figure 32–3.

Aplastic Anemia. *Bone marrow aplasia* means lack of a functioning bone marrow. For instance, a person exposed to gamma ray radiation from a nuclear bomb blast is likely to sustain complete destruction of bone marrow, followed in a few weeks by lethal anemia. Likewise, excessive x-ray treatment, certain industrial chemicals, and even drugs to which the person might be sensitive can cause the same effect.

Megaloblastic Anemia. From the earlier discussion of vitamin B_{12}, folic acid, and intrinsic factor from the stomach mucosa, one can readily understand that loss of any one of these factors can lead to slow reproduction of the erythroblasts in the bone marrow. As a result, these grow too large, with odd shapes, and are called *megaloblasts.* Thus, atrophy of the stomach mucosa, as occurs in *pernicious anemia,* or loss of the entire stomach as the result of surgical total gastrectomy can lead to megaloblastic anemia. Also, patients who have intestinal sprue, in which folic acid, vitamin B_{12}, and other vitamin B compounds are poorly absorbed, often develop megaloblastic anemia. Because the erythroblasts cannot proliferate rapidly enough to form normal numbers of red blood cells, the cells that are formed are mostly oversized, have bizarre shapes, and have fragile membranes. These cells rupture easily, leaving the person in dire need of an adequate number of red cells.

Hemolytic Anemia. Different abnormalities of the red blood cells, many of which are hereditarily acquired, make the cells fragile, so that they rupture easily as they go through the capillaries, especially through the spleen. Even though the number of red blood cells formed is normal, or even much greater than normal in some hemolytic diseases, the red cell life span is so short that cells are destroyed much faster than they can be formed, and serious anemia results. Some of these types of anemia are the following.

In *hereditary spherocytosis,* the red cells are very small and *spherical,* rather than being biconcave discs. These cells cannot withstand compression forces because they do not have the normal loose, baglike cell membrane structure of the biconcave discs. On passing through the splenic pulp and some other tissues, they are easily ruptured by even slight compression.

In *sickle cell anemia,* which is present in 0.3 to 1.0 per cent of West African and American blacks, the cells contain an abnormal type of hemoglobin called *hemoglobin S,* caused by abnormal beta chains of the hemo-

globin molecule, as explained earlier in the chapter. When this hemoglobin is exposed to low concentrations of oxygen, it precipitates into long crystals inside the red blood cell. These crystals elongate the cell and give it the appearance of being a sickle, rather than a biconcave disc. The precipitated hemoglobin also damages the cell membrane, so that the cells become highly fragile, leading to serious anemia. Such patients frequently go into a vicious circle called a sickle cell disease "crisis," in which low oxygen tension in the tissues causes sickling, which causes ruptured red cells, this in turn causing still further decrease in oxygen tension and still more sickling and red cell destruction. Once the process starts, it progresses rapidly, leading to serious decrease in red blood cell mass within a few hours and, often, death.

In *erythroblastosis fetalis,* Rh-positive red blood cells in the fetus are attacked by antibodies from an Rh-negative mother. These antibodies make the Rh-positive cells fragile, leading to rapid rupture and causing the child to be born with serious anemia. This is discussed in Chapter 35 in relation to the Rh factor of blood. The extremely rapid formation of new red cells to make up for the destroyed cells in erythroblastosis fetalis causes a large number of early *blast* forms of red cells to be released into the blood.

EFFECTS OF ANEMIA ON THE CIRCULATORY SYSTEM

The viscosity of the blood, which was discussed in Chapter 14, depends almost entirely on the blood concentration of red blood cells. In severe anemia, the blood viscosity may fall to as low as 1.5 times that of water rather than the normal value of about 3. This decreases the resistance to blood flow in the peripheral blood vessels so that far greater than normal quantities of blood then flow through the tissues and return to the heart. Moreover, hypoxia resulting from diminished transport of oxygen by the blood causes the peripheral tissue vessels to dilate, allowing still further increase in return of blood to the heart, increasing the cardiac output to a still higher level, sometimes to levels three to four times normal. Thus, one of the major effects of anemia is greatly *increased pumping workload on the heart.*

The increased cardiac output in anemia partially offsets the reduced oxygen-carrying effect of anemia because even though each unit quantity of blood carries only small quantities of oxygen, the rate of blood flow may be increased enough so that almost normal quantities of oxygen are actually delivered to the tissues. However, when this same person with anemia begins to exercise, the heart is not capable of pumping much greater quantities of blood than it is already pumping. Consequently, during exercise, which greatly increases tissue demand for oxygen, extreme tissue hypoxia results, and acute cardiac failure ensues.

POLYCYTHEMIA

Secondary Polycythemia. Whenever the tissues become hypoxic because of too little oxygen in the atmosphere, such as at high altitudes, or because of failure of delivery of oxygen to the tissues, as occurs in cardiac failure, the blood-forming organs automatically produce large quantities of extra red blood cells. This condition is called *secondary polycythemia,* and the red cell count commonly rises to 6 to 7 million/mm^3, about 30 per cent above normal.

A common type of secondary polycythemia, called *physiologic polycythemia,* occurs in natives who live at altitudes of 14,000 to 17,000 feet. The blood count is generally 6 to 7 million/mm^3; this is associated with the ability of these people to perform high levels of continuous work even in a rarefied atmosphere.

Polycythemia Vera (Erythremia). In addition to those people who have physiologic polycythemia, others have a pathological condition known as *polycythemia vera,* in which the red blood cell count may be 7 to 8 million/mm^3 and the hematocrit 60 to 70 per cent. Polycythemia vera is caused by a genetic aberration that occurs in the hemocytoblastic cell line that produces the blood cells. The blast cells no longer stop producing red cells when too many cells are already present. This causes excess production of red blood cells in the same manner that a tumor of a breast causes excess production of a specific type of breast cell. It usually causes excess production of white blood cells and platelets as well.

In polycythemia vera, not only does the hematocrit increase, but the total blood volume also increases, on some occasions to almost twice the normal level. As a result, the entire vascular system becomes intensely engorged. In addition, many of the capillaries become plugged by the viscous blood because the viscosity of the blood in polycythemia vera sometimes increases from the normal of 3 times the viscosity of water to 10 times that of water.

Effect of Polycythemia on the Circulatory System

Because of the greatly increased viscosity of the blood in polycythemia, the flow of blood through the vessels is often very sluggish. In accordance with the factors that regulate the return of blood to the heart, as discussed in Chapter 20, increasing the viscosity tends to *decrease* the rate of venous return to the heart. Conversely, the blood volume is greatly increased in polycythemia, which tends to *increase* the venous return. Actually, the cardiac output in polycythemia is not far from normal because these two factors more or less neutralize each other.

The arterial pressure is also normal in most people with polycythemia, though in about one third of them the arterial pressure is elevated. This means that the

blood pressure-regulating mechanisms can usually offset the tendency for increased blood viscosity to increase peripheral resistance and, thereby, increase arterial pressure. Beyond certain limits, however, these regulations fail.

The color of the skin depends to a great extent on the quantity of blood in the skin subpapillary venous plexus. In polycythemia vera, the quantity of blood in this plexus is greatly increased. Furthermore, because the blood passes sluggishly through the skin capillaries before entering the venous plexus, a larger than normal quantity of hemoglobin is deoxygenated. The blue color of all this deoxygenated hemoglobin masks the red color of the oxygenated hemoglobin. Therefore, a person with polycythemia vera ordinarily has a ruddy complexion with a bluish (cyanotic) tint to the skin.

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CHAPTER 27

THE RED CELL MEMBRANE

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The easy accessibility of the red cell has allowed the human erythrocyte membrane to become the most thoroughly studied biologic membrane. It is composed of three major structural elements: a lipid bilayer primarily composed of phospholipids and cholesterol; integral proteins embedded in the lipid bilayer that span the membrane; and a membrane skeleton on the internal side of the red cell membrane. The erythrocyte membrane has many important functions. The lipid bilayer provides an impermeable barrier between the cytoplasm and the external environment and helps maintain a slippery exterior so that erythrocytes do not adhere to endothelial cells or aggregate and occlude the micro-circulation. The red cell membrane and its skeleton provide the erythrocyte with its unique deformability, durability, and tensile strength to undergo large deformations during repeated passages through narrow microcirculatory channels. The erythrocyte membrane also assembles and organizes the proteins of the lipid bilayer and the underlying skeleton. This allows the red cell to participate in a wide range of functions. These include influencing cellular metabolism by selectively and reversibly binding and inactivating glycolytic enzymes, retaining organic phosphates and other vital compounds, removing metabolic waste, and sequestering the reductants required to prevent corrosion by oxygen. During erythropoiesis, the membrane imports the iron required for the synthesis of hemoglobin. At the level of the organism, the membrane participates in the maintenance of pH homeostasis, participating in the exchange of chloride and bicarbonate. Investigation of disorders of the erythrocyte membrane such as hereditary spherocytosis, elliptocytosis, and pyropoikilocytosis has advanced our understanding of the normal structure/function relationships of the membrane as well as providing us with an understanding of the inheritance and expression of these disorders.

INTRODUCTION

The erythrocyte membrane accounts for only 1 percent of total weight of the red cell, yet it plays an integral role in the maintenance of erythrocyte integrity. The red cell membrane and its skeleton provide the erythrocyte the flexibility, durability, and tensile strength to undergo large deformations during repeated passages through narrow microcirculatory channels. The red cell membrane maintains a slippery exterior so that erythrocytes do not adhere to endothelial cells or aggregate and occlude the microcirculation. The membrane plays an important role in metabolism by selectively and reversibly binding and inactivating glycolytic enzymes. The membrane retains organic phosphates and other vital compounds and helps remove metabolic waste. The membrane sequesters the reductants required to prevent corrosion by oxygen. During erythropoiesis, the membrane responds to erythropoietin and imports the iron required for the synthesis of hemoglobin. At the level of the organism, the membrane participates in the maintenance of pH homeostasis, participating in the exchange of chloride and bicarbonate.

The easy accessibility of the human erythrocyte membrane has allowed it to become the most thoroughly studied biologic membrane. Erythrocytes are the cells about which the most detailed information is available concerning the normal structure and function of their membrane and about the molecular pathology of disorders due primarily to abnormal membrane or cytoskeletal structure. The erythrocyte membrane remains the paradigm for ongoing studies of other cell types. Although the primary structure (Fig. 27-1) and a number of the important functions of the red cell membrane are known, its study continues to yield important insights into our understanding of membrane structure and function. Genetic investigation of disorders of the erythrocyte membrane has advanced our understanding of the normal structure/function relationships of the membrane as well as provided us with an understanding of the inheritance and expression of these disorders.

COMPOSITION OF THE ERYTHROCYTE MEMBRANE

The erythrocyte membrane is composed of three major structural elements: a lipid bilayer primarily composed of phospholipids and cholesterol that provides a permeability barrier between the external environment and the red cell cytoplasm; integral proteins embedded in the lipid bilayer that span the membrane; and a membrane skeleton on the internal side of the red cell membrane that provides structural integrity to the cell.

MEMBRANE LIPIDS

COMPOSITION

Lipids comprise 50 to 60 percent of red cell membrane mass. The principal membrane lipids are phospholipids and cholesterol, which are present in nearly equal amount.[1] Small amounts of glycolipids, primarily globoside, are also present. The primary phospholipids are phosphatidylcholine (28 percent of total phospholipids), phosphatidylethanolamine (27 percent), sphingomyelin (26 percent), phosphatidylserine (13 percent), and phosphatidylinositol.

Membrane phosphoinositides are phospholipids that contain phosphatidylinositol (PI) or its phosphorylated forms, PI-4-monophosphate and PI-4,5-biphosphate (PIP and PIP-2 respectively). In nucleated cells, phosphoinositides are precursors of important intracellular second messengers such as inositol-1,4,5-triphosphate and diacylglycerol that participate in regulation of many cellular processes. In mature erythrocytes, phosphoinositides represent 2 to 5 percent of total phospholipids, residing largely at the inner membrane surface and undergoing rapid phosphorylation and dephosphorylation. In red cells, they are involved in regulation of calcium transport and interaction of transmembrane and skeletal proteins (e.g., glycophorin C and protein 4.1), and they have been proposed to participate in the control of the discocyte-echinocyte shape transformation.[2]

In the erythrocyte, cholesterol is present in a free, unesterified form, and it is almost entirely hydrophobic. Its primary role appears to be to control membrane fluidity even under conditions that might lead to phospholipid crystallization and rigidification of the bilayer.

Acronyms and abbreviations that appear in this chapter include: AE1, anion exchanger-1; AQP1, aquaporin-1; ATP, adenosine 5′-triphosphate; HE, hereditary elliptocytosis; LCAT, lecithin-cholesterol acyltransferase; MAGUK, membrane-associated guanylate kinase; PAS, periodic acid-Schiff; PI, phosphatidylinositol; PIP, PI-4-monophosphate; PIP-2, PI-4,5-biphosphate; SDS, sodium dodecyl sulfate.

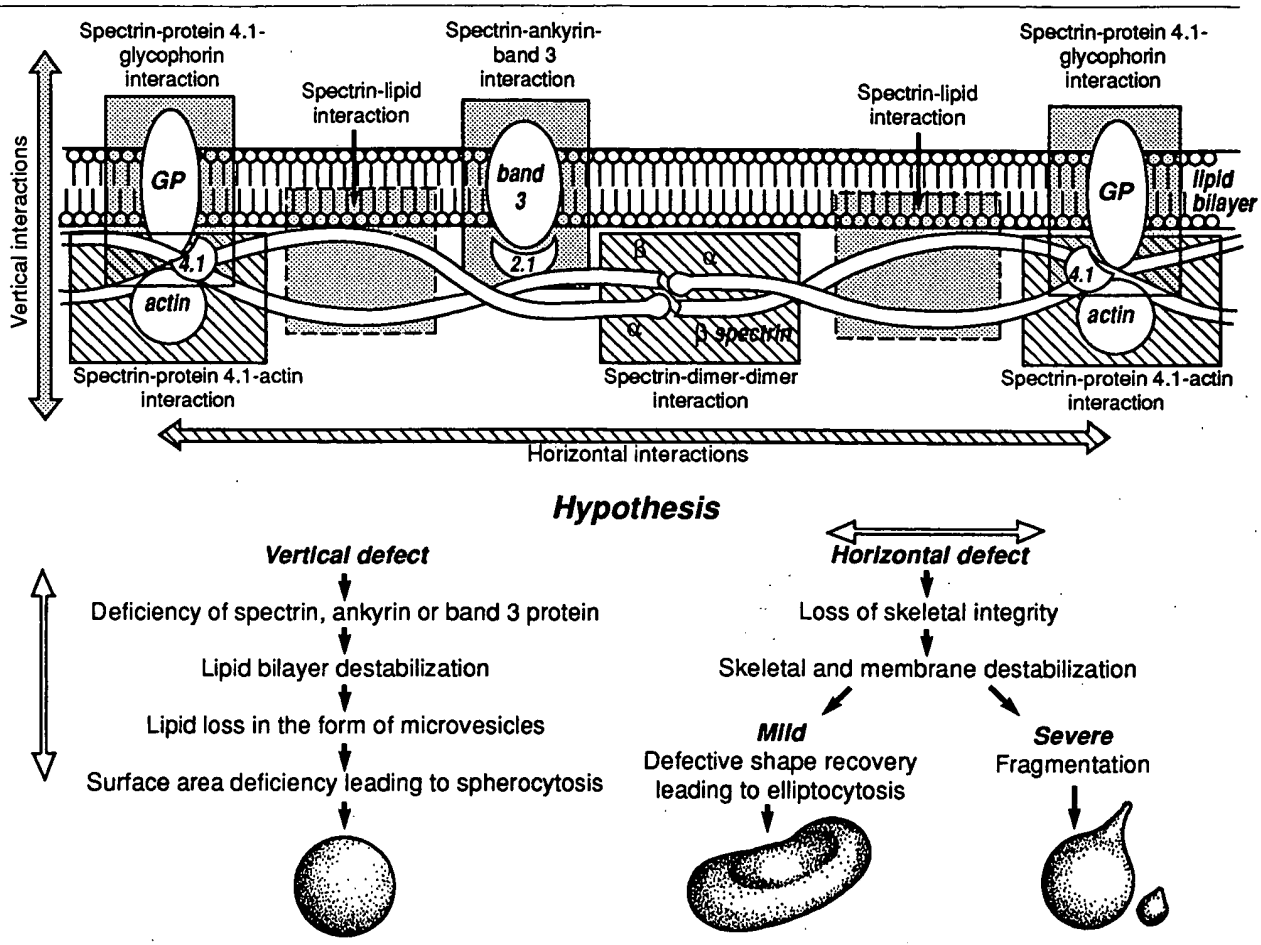

FIGURE 27-1 A schematic diagram illustrating the molecular assembly of the major erythrocyte membrane proteins and a model of the principal molecular defect in hereditary spherocytosis (HS), elliptocytosis (HE), and pyropoikilocytosis (HPP). Membrane protein-protein and protein-lipid associations can be divided into two categories: *(1) vertical interactions*, which are perpendicular to the plane of the membrane and involve spectrin-ankyrin-band 3 interaction, spectrin-protein 4.1-glycophorin C connection, and weak interactions between spectrin and the negatively charged lipids of the inner half of the membrane lipid bilayer, and *(2) horizontal interactions*, which are parallel to the plane of the membrane.

MEMBRANE LIPID DISTRIBUTION

Phospholipids are asymmetrically distributed in the red cell membrane with phosphatidylserine and phosphatidylethanolamine primarily in the inner hemileaflet, while sphingomyelin and phosphatidylcholine are outwardly oriented. This asymmetric distribution of phospholipids is a dynamic system involving a constant exchange ("flip-flop")[3] between the phospholipids of the two-bilayer leaflets. Maintenance of this asymmetry appears to be important in the regulation of hemostasis, as PS on the outer leaflet provides a site for prothrombinase binding, causing the red cell surface to become prothrombotic. Phospholipid flipping may contribute to the occurrence of thromboses in a variety of disorders including sickle cell disease (see Chap. 47) and diabetes.[4] The presence of PS on the outer surface of the red cell is one of the earliest changes in apoptosis, and it has been correlated with complement activation and red cell clearance by macrophages.

Enzymes called *flippases* actively translocate PS and PE to the inner leaflet; *floppases* catalyze translocation to the outer leaflet. Asymmetry seems to depend on the fact that flipping occurs at a higher rate than flopping. Flippase activity is mediated by a 130-kDa integral membrane protein that is a member of the Mg^{++}-dependent, P-glycoprotein ATPases.[5] Floppase activity in red cell membranes appears to be mediated by the multidrug resistance protein 1 (MRP1).[6,7]

A *scramblase* activated by elevated intracellular calcium that promotes randomization and loss of asymmetry has been isolated and cloned.[8,9] This scramblase mediates redistribution of membrane phospholipids in activated, injured, or apoptotic cells and is activated by calcium.[10,11] Derangements within the red cell often raise intracellular calcium by direct or indirect damage to ion channels and pumps. Scott's syndrome is a congenital bleeding disorder in which red cells and platelets expose subnormal amounts of PS on the outer surface in response to calcium, but it does not appear to be due to scramblase deficiency.[12,13]

Glycolipids and cholesterol are intercalated between the phospholipids in the bilayer with their long axes perpendicular to the bilayer plane. Red cell glycolipids are located entirely in the external half of the bilayer with their carbohydrate moieties extending into the aqueous phase. They carry several important red cell antigens, including A, B, H, and P, and may serve other important functions. The location of membrane cholesterol is less certain, but it appears that cholesterol is present in about equal proportions on both sides of the bilayer.

LIPID SYNTHESIS AND RENEWAL

The synthesis and assembly of red cell membrane lipids takes place during erythropoiesis. Mature erythrocytes are unable to synthesize fatty acids, phospholipids, or cholesterol de novo and depend on lipid exchange and fatty acid acylation for phospholipid repair and renewal.

These renewal pathways, although limited, permit a slow replacement of membrane lipid components.

Lipid exchange rates vary considerably. The exchange of unesterified cholesterol takes place in several hours, while the outer bilayer phospholipid phosphatidylcholine and sphingomyelin exchange with the phospholipids of plasma lipoproteins over a period of days. Because of their inaccessibility, the inner bilayer phospholipids phosphatidylserine and phosphatidylethanolamine are unable to participate in lipid exchange. Unesterified membrane cholesterol exchanges readily with the unesterified cholesterol in plasma lipoproteins where it is partially converted to esterified cholesterol by lecithin-cholesterol acyltransferase (LCAT). Because the newly formed cholesteryl ester cannot return to the red cell membrane, LCAT catalyzes a unidirectional pathway that depletes the membrane of cholesterol and decreases its surface area, and there is virtually no esterified cholesterol in the membrane. This process is reversed when this enzyme is absent or inactive, leading to a net accumulation of free cholesterol in the cells.

In addition to passive exchange, free fatty acids can be incorporated into red cell phospholipids in a two-step reaction requiring lysophospholipid, ATP, magnesium, and coenzyme A. Following the acyl-coenzyme A formation, the fatty acid is incorporated into the lysophospholipid at the inner bilayer leaflet. This pathway also participates in the maintenance of phospholipid asymmetry, as evidenced by a rapid outward translocation of the newly synthesized phosphatidylcholine. Although this pathway consumes a small amount of energy, it may be important for detoxification of naturally formed lysophosphatides in the cells, as evidenced by their gradual accumulation during ATP depletion.

LIPID BILAYER FLUIDITY

Under physiologic conditions, the lipid bilayer is in a liquid state, allowing both the transmembrane proteins and the cell surface molecules (such as surface antigens) to move in the plane of the membrane. Lipid bilayer fluidity is influenced by several factors including: (1) temperature, which determines the phase transition between a liquid state and gel state; (2) free cholesterol content, as the rigid sterol ring of cholesterol decreases lipid bilayer fluidity; and (3) the length and the degree of phospholipid fatty acid saturation. Saturated fatty acids with a relatively rigid backbone resist motion, while the unsaturated fatty acids have relatively unrestricted movements, thereby increasing the fluidity of the lipid bilayer. Because of the differences in the composition of phospholipids between the two-bilayer halves, the bilayer is asymmetric in terms of the fluidity of the two hemileaflets.

MEMBRANE PROTEINS

Several general observations can be made about erythrocyte membrane proteins. Most of these proteins also are present in nonerythroid cells, where they fulfill similar functions. Many of these proteins are members of super families of proteins that are structurally related but genetically distinct. This genetic diversity explains why the clinical expression of many (but not all) red cell membrane protein mutations is confined to the erythroid lineage. Tissue- and developmental stage-specific alternative splicing or the usage of alternate initiation codons or alternate promoters creates multiple isoforms of many of these proteins. Finally, many are large, multifunctional proteins. As a result, mutations within a given region of the protein may lead to distinct differences in abnormalities of function and clinical phenotype.

Membrane proteins are classified according to the ease with which they can be removed from whole red cell membrane preparations in the laboratory. Integral proteins are firmly embedded into or through the lipid bilayer by hydrophobic domains within their amino acid sequences; only harsh reagents such as detergents can extract them. Peripheral proteins are more loosely associated; they are extractable by high- or low-salt or high-pH extraction. Peripheral proteins are attached indirectly to the lipid bilayer by means of covalent or noncovalent binding to the (usually) cytoplasmic domains of embedded or anchored proteins and typically are associated with only one face of the membrane (i.e., exterior or extracellular versus interior or cytoplasmic), whereas many integral proteins often protrude into both spaces. The affinity with which proteins associate with the membrane is not a static property. Rather, proteins can become more or less tightly bound according to their state of phosphorylation, methylation, glycosylation, or lipid modification (myristylation, palmitylation, or farnesylation).[2]

Fairbanks and colleagues assigned names to the proteins extracted from red cell membranes (Fig. 27-1 and Table 27-1).[14] These designations were based on their mobility in a sodium dodecyl sulfate (SDS)-acrylamide gel system; the slowest migrating band was band (or protein) 1, the next slowest band, band 2, etc. Subbands were designated with decimals. After further analysis, some of these proteins, such as bands 1 and 2, alpha and beta spectrin, were renamed. Other proteins, such as protein 4.1, were never renamed.

INTEGRAL MEMBRANE PROTEINS

Band 3 Band 3 (anion exchanger-1, AE1) is an abundant (10^6 copies per cell) transmembrane glycoprotein with a molecular mass of about 100 kDa. It serves as a regulator of ion content, red cell deformability, intermediary metabolism, and red cell senescence.[15,16] The NH_2-terminus of the protein encodes a 43-kDa cytoplasmic domain with COOH-terminus of the protein folded into helices and β sheets to form the membrane-spanning domain. The region between the NH_2-terminus and the first membrane-spanning segment forms an interhinge domain.

Band 3 is the major anion (chloride-bicarbonate) exchanger of the red cell. It regulates metabolic pathways by sequestering key pathway enzymes, such as the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and aldolase, as well as carbonic anhydrase II. Band 3 contains important binding sites for interaction with other membrane proteins including ankyrin, protein 4.1, protein 4.2, and possibly spectrin.[17,18] Binding of the cytoplasmic domain to ankyrin is a critical mechanism for attachment of the membrane skeleton to the plasma membrane, and the interdomain hinge at this attachment point may be a crucial determinant of the flexibility or rigidity of the erythrocyte.[19]

The Glycophorins Glycophorins are the most abundant integral membrane glycoproteins in erythrocytes, and, because of their high sialic acid content, they account for more than 95 percent of the periodic acid–Schiff (PAS)-staining capacity of erythrocytes.[20] The glycophorins are 0-glycosylated and are composed of a single extracellular hydrophilic NH_2-terminal domain, a single membrane-spanning domain, and a COOH-terminal cytoplasmic tail. Characterization of cDNA and genomic clones encoding the glycophorins has revealed that they fall into two distinct subgroups.[21] Glycophorins A and B are homologous to each other and are encoded by two closely linked genes. Glycophorins C and D arise from a single locus bearing no particular homology to the genes for glycophorins A and B. Glycophorin D differs from glycophorin C by use of an alternate translation start site created by alternative splicing. Another gene linked in tandem with those for glycophorins A and B, glycophorin E, has been cloned, but no protein product has been identified.[22]

The functional roles of the glycophorins are beginning to be revealed. Because the glycophorins constitute more than 60 percent of the net negative surface charge of red cells, they may modulate red cell–red cell and red cell–endothelial cell interactions. GPC, which interacts in a complex with protein 4.1 and p55, plays a critical role in regulating the stability, deformability, and shape of the membrane. GPC deficiency leads to elliptocytic erythrocytes that are less stable

TABLE 27-1 MAJOR RED CELL MEMBRANE PROTEINS

Band	Protein	M_r (gel)	M_r (calc)	Copies Per Cell ($\times 10^3$)	(%) of total[a]	Gene Symbol	Chromosomal Localization	Amino Acids	Gene Size, kb	# of Exons	Involvement in Hemolytic Anemias
1	α Spectrin	240	280	240	16	*SPTA1*	1q22-q23	2429	80	52	HE, HS
2	β Spectrin	220	246	240	14	*SPTB*	14q23-q24.2	2137	>100	32	HE, HS
2.1	Ankyrin[b]	210	206	120	4.5	*ANK1*	8p11.2	1881	>100	40	HS
2.9	α Adducin[c]	103	81	30	2	*ADDA*	4p16.3	737	85	16	N
2.9	β Adducin[c]	97	80	30	2	*ADDB*	2p13-2p14	726	~100	17	N
3	Anion exchanger-1	90–100	102	1200	27	*EPB3*	17q21-qter	911	17	20	HS, SAO, HAc
4.1	Protein 4.1	80	66	200	5	*EL11*	1p33-p34.2	588[d]	>100	23	HE
4.2	Pallidin	72	77	200	5	*EB42*	15q15-q21	691	20	13	HS
4.9	Dematin[e]	48 + 52	43	40[f]	1	*EPB49*	8p21.1	383	—	—	N
4.9	p55[e]	55	53	80	—	*MPP1*	Xq28	466	—	—	N
5	β-Actin	43	42	400–500	5.5	*ACTB*	7pter-q22	375	>4	6	N
5	Tropomodulin	43	41	30		*TMOD*	9q22	359	—	—	N
6	G-3P-D[g]	35	37	500	3.5[g]	*GAPD*	12p13.31-p13.1	335	5	9	N
7	Stomatin	31	32	—	2.5	*EPB72*	9q33-q34	288	12	7	HSt
7	Tropomyosin	27 + 29	28	80	1	*TPM3*	1q31	239	—	—	N
PAS-1	Glycophorin A[h]	36		500–1000	85	*GYPA*	4q28-q31	131	>40	7	HE
PAS-2	Glycophorin C[h]	32	14	50–100	4	*GYPC*	2q14-q21	128	14	4	HE
PAS-3	Glycophorin B[h]	20		100–300	10	*GYPB*	4q28-q31	72	>30	5	N
	Glycophorin D[h]	23		20	1	*GYPD*	2q14-q21	107	14	4	N
	Glycophorin E					*GYPE*	4q28-q31	59	>30	4	N

[a]Quantitation based on scanning of SDS-PAGE gels of red cell membranes prepared from healthy blood donors. For glycophorins, the values indicate the fraction of PAS-positive material.
[b]Bands 2.1, 2.2, 2.3, and 2.6 are protein isoforms of erythroid ankyrin, at least some of which are produced by alternative splicing of ankyrin mRNA.
[c]Since adducin comigrates with band 3, no numerical band designation is available.
[d]Numerous erythroid and nonerythroid isoforms of protein 4.1 produced by alternative splicing have been described. The values correspond to the major erythroid protein 4.1 isoform.
[e]Both dematin and p55 migrate within the 4.9 band.
[f]40,000 of dematin trimers are present in one red cell.
[g]Variable amounts of band 6 are detected in red cell membranes.
[h]Detectable on PAS-stained gels only.
NOTE: HS, hereditary spherocytosis; HE, hereditary elliptocytosis; HPP, hereditary pyropoikilocytosis; SAO, Southeast Asian ovalocytosis; HAc, hereditary acanthocytosis; HSt, hereditary stomatocytosis; G-3-PD, glyceraldehyde-3-phosphate dehydrogenase; N, no hematologic abnormalities reported.

and less deformable than normal red cells. The glycophorins play important roles in clinical immunohematology, carrying a number of blood group antigens including MN, Ss, Miltenberger V, En(a-), M^KM^k, and Gerbich (see Chap. 137).

Other Integral Membrane Proteins The red cell membrane contains other integral membrane proteins including the Rh D protein (see Chap. 137) and various ion pumps and channels (see below).

PERIPHERAL MEMBRANE PROTEINS

The major proteins of the erythrocyte membrane skeleton are spectrin; ankyrin; actin; proteins 4.1, 4.2, and 4.9; p55; and the adducins. These proteins form an interlocking network that attaches to the inner face of the membrane primarily by binding to the cytoplasmic domains of band 3 and the glycophorins.

Spectrin Spectrin is the most abundant and largest protein of the erythrocyte membrane skeleton, constituting 75 percent of its mass and present at a concentration of about 200,000 molecules per cell.[23] Spectrin is composed of two subunits, α and β, that despite many similarities are structurally distinct and are encoded by separate genes (Fig. 27-2a).[24,25] Both α and β spectrin contain homologous 106 amino acid repeats that are folded into α-helical segments containing three antiparallel helices connected by short nonhelical segments.[26,27] The presence of spectrin repeats suggests that spectrin evolved from the duplication of a single ancestral gene.[28]

The fundamental structure of the spectrin molecule is that of $\alpha\beta$ heterodimers that align and intertwine with each other in antiparallel fashion with respect to their NH_2-termini to form flexible, rodlike molecules (Fig. 27-2a).[26,29] These dimers further self-associate to form tetramers and higher-order oligomers. These tetramers, composed of multiple repeats, provide a strong, elastic, rodlike filament that associates into multimolecular complexes capable of lending shape and resiliency to the overlying plasma membrane via formation of a lattice-like meshwork linked to integral membrane proteins.[30] Direct interactions of a weaker nature may also occur between spectrin filaments and the lipid bilayer itself. The side-to-side assembly of α- and β-spectrin chains in a zipper-like fashion begins at a defined nucleation site composed of four repeats from each chain, α19 to α22 and β1 to β4 respectively.[31,32] After tight association of complementary nucleation sites, a conformational change is initiated that promotes pairing of the remainder of the two chains. A common α-spectrin variant, α^{LELY}, interferes with normal nucleation and decreases the synthesis of functionally competent spectrin chains and may influence clinical expression of spectrin mutations (see Chap. 43).[33]

The NH_2-terminus of α spectrin and the COOH-terminus of β spectrin are the regions involved in $\alpha\beta$ heterodimer self-association.[29] Spectrin also binds to actin and protein 4.1 via the NH_2-terminus of β spectrin and ankyrin via sites in repeats β15 and β16 near the COOH-terminus.[34–36] Other nonrepeat sequences in spectrin provide the recognition sites for binding to other modifiers, including kinases and calmodulin.

The functions of spectrin are to maintain cellular shape, regulate the lateral mobility of integral membrane proteins, and provide structural support for the lipid bilayer.[23] Defects in the $\alpha\beta$ self-association site are associated with hereditary elliptocytosis and hereditary pyropoikilocytosis (see Chap. 43). Compound heterozygosity or homozgosity for defects outside the $\alpha\beta$ self-association site are associated with severe, recessively inherited spherocytosis.

Ankyrin Ankyrin is an asymmetric polar protein that can be separated into three functional domains by mild proteolysis: an NH_2-terminal membrane-binding domain that contains sites for band 3 and other ligands, a central domain that contains sites for spectrin binding, and a COOH-terminal "regulatory" domain that influences ankyrin-

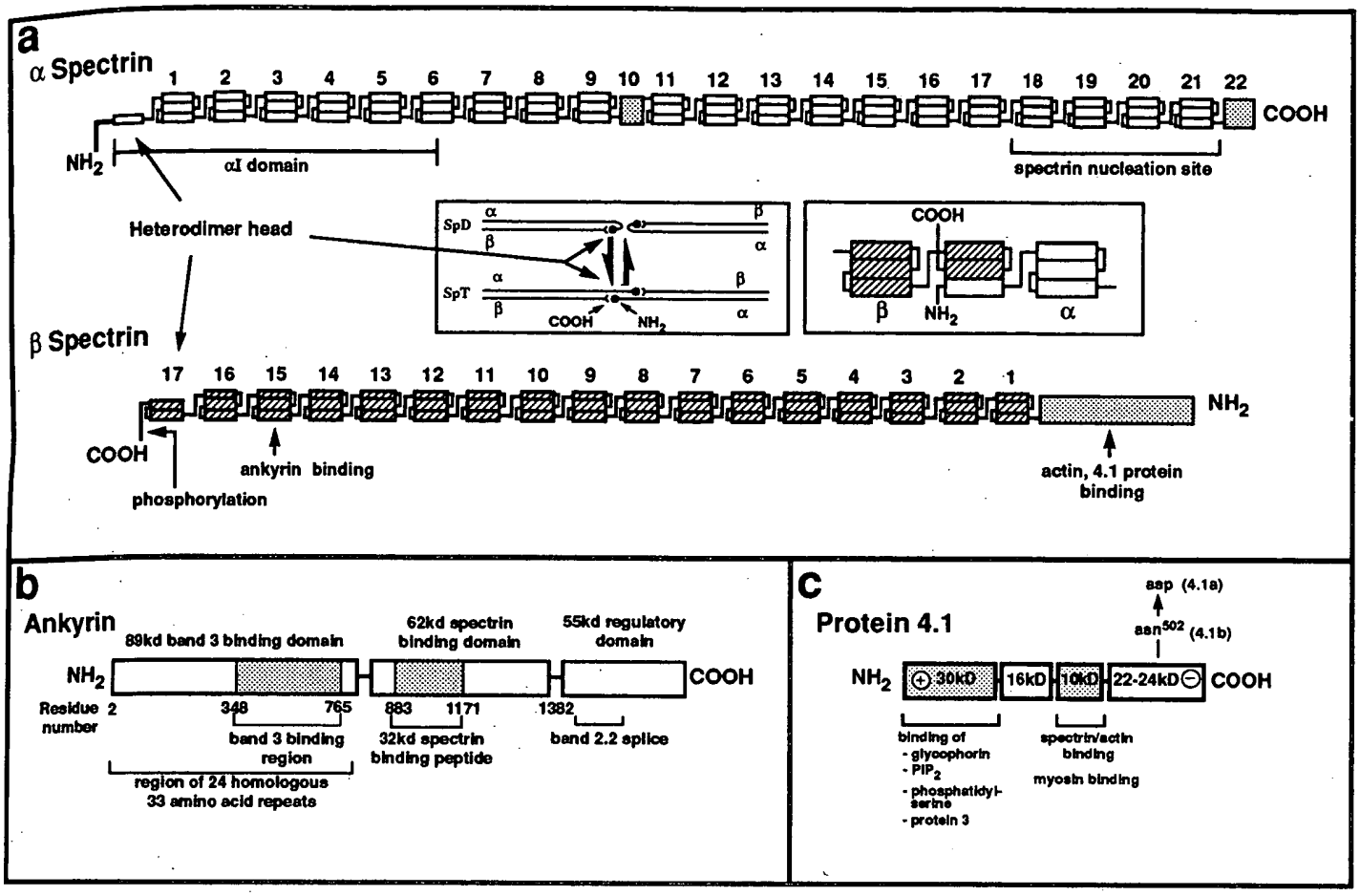

FIGURE 27-2 Spectrin, ankyrin, and protein 4.1. *(a) α and β spectrin.* Both proteins are composed of multiple homologous triple helical repetitive segments, numbered starting from the NH_2-terminus. α spectrin and β spectrin are shown in antiparallel orientation, their configuration in the spectrin heterodimer. Stippled regions represent nonhomologous segments. The αI domain (a tryptic peptide of α spectrin involved in association of α and β spectrin), the spectrin nucleation site, and the ankyrin, actin, and protein 4.1 protein-binding sites are shown. In the head region of spectrin, α and β spectrin interact to form either a heterodimer (SpD) or tetramer (SpT). The contact site between the α and β chains of a spectrin heterodimer or the opposed α and β chains of the tetramer is formed by a combined αβ triple helical segment (*insert*). *(b) Ankyrin.* The three major functional and structural domains, as defined by limited proteolytic digestion, are shown. The band 3 and spectrin-binding regions are shaded. The regulatory domain is subject to extensive alternative splicing, including the band 2.2 splice, which produces an activated form of ankyrin. *(c) Protein 4.1.* The four major functional and structural domains, as defined by limited proteolytic digestion, are shown. The regions where the 4.1 protein binds to other membrane proteins are shaded. The protein 4.1a isoform is derived from the 4.1b isoform by deamidation of aspartic acid 508 (see text for details).

protein interactions (Fig. 27-2b).[23,37,38] The membrane-binding domain contains 24 tandem repeats called *cdc10/ankyrin repeats* that contain multiple protein-binding sites.[39] Ankyrin repeats are highly conserved, L-shaped structures composed of a pair of α-helices that form an antiparallel coiled-coil, followed by an extended loop perpendicular to the helices and a β hairpin.[40] These repeats have been found in proteins with a wide variety of functions.[41,42] The regulatory domain consists of multiple isoforms generated by alternative splicing.[43,44] One of these isoforms (ankyrin 2.2) enhances ankyrin binding to band 3 and spectrin.[43]

Ankyrin provides the primary linkage between the membrane skeleton via spectrin binding and the lipid bilayer via band 3 binding (Fig. 27-1). Disruption of either of these linkages significantly decreases membrane stability. Ankyrin also appears to be involved in the local segregation of integral membrane proteins within function domains on the plasma membrane. The importance of ankyrin in the maintenance of membrane stability is underscored by the observation that abnormalities of ankyrin are the most common cause of typical hereditary spherocytosis (see Chap. 43).

Protein 4.1 Protein 4.1 is a phosphoprotein that can be separated by mild chymotryptic digestion into four proteolytic domains: 30 kDa, 16 kDa, 10 kDa, and 22 to 24 kDa (Fig. 27-2c). In red cells, two molecular weight forms are found, protein 4.1a and protein 4.1b, with protein 4.1a predominating in older erythrocytes. Protein 4.1a is derived from protein 4.1b by the gradual deamidation of two Asn residues in a nonenzymatic, age-dependent manner.[45] Alternative splicing leads to the production of a large number of tissue- and developmental stage-specific protein 4.1 isoforms,[46-50] e.g., alternatively spliced isoforms of the 10-kDa domain contain the spectrin-actin-binding site and provide erythroid and stage-specific specificity.[47-49] Protein 4.1 utilizes two different initiation codons. The upstream initiation codon encodes a protein of 135 kDa found in most nonerythroid cells.[50] The downstream initiation codon encodes the 85-kDa protein found primarily in erythrocytes.

The primary role of protein 4.1 is in the linkage of the spectrin-actin membrane skeleton to the lipid bilayer by facilitating complex formation between spectrin-actin fibers, the cytoplasmic domain of band 3, and p55/GPC (Fig. 27-1).[51] Qualitative or quantitative defects

of protein 4.1 lead to hereditary elliptocytosis (HE) with concomitant GPC and p55 deficiency (see Chap. 43). HE-related protein 4.1 mutations have included variants that affect protein 4.1 alternative splicing and initiation codon usage. Interestingly, mice with targeted disruption of the protein 4.1 gene demonstrate, in addition to hematologic effects, subtle neurologic abnormalities.[52] The applicability of this observation to humans with defects of protein 4.1 is unknown.

Protein 4.2 Protein 4.2 is a member of the transglutaminase family of proteins.[53] However, protein 4.2 does not possess transglutaminase activity as it lacks a critical residue in the active transglutaminase site. There are at least four isoforms of protein 4.2 created by alternative splicing; the functional significance of these is not known.[54] Protein 4.2 binds to several proteins, including band 3, protein 4.1, ankyrin, and ankyrin-protein 3 complexes. The major function of protein 4.2 is to stabilize spectrin-actin-ankyrin association with band 3.[55] It may also protect the membrane skeleton from premature aging by binding calcium and other cofactors that normally activate red cell transglutaminases, as these transglutaminases would otherwise cross-link proteins and lead to their inactivation. Deficiency of protein 4.2 has been associated with recessively inherited hereditary spherocytosis (see Chap. 43). Erythrocytes from mice with targeted inactivation of the protein 4.2 gene are dehydrated spherocytes with altered cation content (increased K^+/decreased Na^+).[56]

p55 Protein p55 is a phosphoprotein member of the MAGUK (membrane-associated guanylate kinase) family of proteins.[57] Homologues of p55 include signal transduction proteins, tumor suppressor genes, and proteins important in cell-cell interactions. p55 binds to protein 4.1 through a binding motif in the COOH-terminal MAGUK domain and to GPC via a PDZ motif.[58] A primary deficiency state for p55 has not been described, possibly because it is a widely expressed protein, and it may play a critical role in protein-protein interactions in other tissues. Deficiency of protein 4.1 or GPC lead to concomitant p55 deficiency. Studies of this interesting protein may shed important light on mechanisms whereby the erythrocyte membrane influences other cellular processes.

Adducin Adducin, a calcium/calmodulin-binding phosphoprotein located at the spectrin-actin junctional complex, is composed of $\alpha\beta$ adducin heterodimers.[59] α and β adducin are structurally similar proteins encoded by separate genes.[60] Adducin contains a "MARCKS" phosphorylation domain that regulates calcium/calmodulin-regulated capping and bundling of actin filaments.[61,62] Adducin promotes the interaction of spectrin and actin and binds and bundles actin filaments.[63,64] A primary deficiency of adducin in human disease has not been described. Mice with targeted inactivation of β adducin suffer from compensated spherocytic anemia, suggesting that the adducins may be candidate genes for recessively inherited spherocytosis.[65]

Other Peripheral Membrane Proteins Dematin (protein 4.9), tropomyosin, proteins related to troponin, and other proteins associated with actin in nonerythroid cells are found in erythrocytes. The functional roles of these proteins are now being revealed. For example, the amount of dematin present in the erythrocyte declines dramatically during erythrocyte maturation suggesting that it may play an important role in cellular maturation.

FUNCTIONS OF THE ERYTHROCYTE MEMBRANE

The roles of the erythrocyte membrane include assembling and organizing proteins of the lipid bilayer and the underlying skeleton, providing the red cell with its unique deformability and stability, participating in membrane biogenesis and aging, and providing an impermeable barrier between the erythrocyte cytoplasm and the external environment.

MEMBRANE ASSEMBLY AND ORGANIZATION

Membrane organization arises from interactions between integral membrane proteins and other molecules contacting the hydrophilic faces of the membrane and by protein-protein or protein-lipid interactions within the bilayer or the underlying membrane skeleton. The avidity of these interactions is modulated by posttranslational modifications of the participating proteins. By utilizing the cytoplasmic domains of embedded proteins as attachment points, the membrane skeleton not only affixes itself to the lipid bilayer but also provides a means to order the topological arrangement of transmembrane proteins.[66] This attachment constrains motion along the transverse plane.

In the intact erythrocyte membrane, the membrane skeleton appears as a lattice-like network, with about 60 percent of the lipid bilayer directly laminated to the underlying membrane skeleton.[67] When skeletal preparations are stretched, the individual skeletal proteins can be visualized as a highly ordered lattice of hexagons. The corners of each hexagon are globular structures called the *junctional complex* composed of complexes of F-actin, along with dematin, adducin, and protein 4.1.[68] Spectrin tetramers form the arms of the hexagons, cross-bridging individual junctional complexes. Spectrin cross-bridges are largely formed by spectrin tetramers, with occasional double tetramers or hexamers. Each spectrin tetramer is composed of two $\alpha\beta$ heterodimers assembled at their "head" regions into tetramers. At their tails, the tetramers bind to junctional complexes of actin, with the aid of protein 4.1 and adducin. The above *horizontal* protein contacts are important in the maintenance of the structural integrity of the cell, accounting for the high tensile strength of the erythrocyte.

The skeleton is affixed to the integral proteins of the membrane by several protein-protein interactions (Fig. 27-1).[23,68,69] Spectrin tetramers are connected to ankyrin, the major skeleton/membrane linkage protein via an interaction site in β spectrin. Ankyrin links the underlying spectrin skeleton to tetramers of band 3, the major transmembrane protein of the red cell. At the distal ends of spectrin tetramers, spectrin binds to the membrane via linkage to protein 4.1, which binds GPC and protein p55. In addition, both spectrin and protein 4.1 bind weakly to phosphatidylserine, which preferentially is located at the inner leaflet of the lipid bilayer. These *vertical* protein-protein and protein-lipid interactions are critical in the stabilization of the lipid bilayer, precluding its loss from the cells.

As discussed in Chap. 43, hereditary spherocytosis is characterized by defects of *vertical* interactions, which lead to uncoupling of the lipid bilayer from the skeleton and a release of membrane microvesicles.[70] In contrast, the principal defects in hereditary elliptocytosis and pyropoikilocytosis involve *horizontal* interactions of membrane skeletal proteins that maintain the two-dimensional integrity of the skeleton.

Red cell membrane proteins are subject to a variety of posttranslational modifications or other regulatory effects including phosphorylation, fatty acid acylation, methylation, glycosylation, deamidation, oxidation, and limited proteolytic cleavage.[2] With the exception of membrane protein phosphorylation, such modifications are relatively static and irreversible. In contrast, membrane protein phosphorylation represents a highly dynamic system of multiple protein kinases and phosphatases that constantly phosphorylate and dephosphorylate serine, threonine, and tyrosine residues, often in an amino-acid-specific and protein-site-specific manner, thereby tightly regulating association of membrane proteins. Additionally, membrane protein associations are influenced by a variety of intracellular factors including calcium and calmodulin, phosphoinositides, and polyanions such as 2,3-bisphosphoglycerate.

The red cell surface is negatively charged, primarily because of a high concentration of neuraminic acid residues. Ninety percent of these residues reside on glycophorin A with the remaining shared by the other glycophorins and band 3. Alterations in erythrocyte surface

charge appear to have deleterious effects on the cell. For example, in sickle red cells, surface charge clustering may play a role in the adhesion of these cells to the surface of endothelial cells.

CELLULAR DEFORMABILITY AND MEMBRANE STABILITY

The most important property of red cells required for normal survival is cellular deformability.[71] *Deformability* refers to the ability of the erythrocyte to undergo distortions and deformations and then to resume its normal shape without fragmentation or loss of integrity. This is best exemplified in the wall of the splenic sinus where red cells squeeze through narrow slits among the endothelial cells that line the splenic sinus wall. The cellular deformability of erythrocytes is determined by three factors: (1) cell geometry (biconcave disc shape); (2) cytoplasmic viscosity, principally determined by the properties and the concentration of hemoglobin in the cells; and (3) intrinsic viscoelastic properties of the red cell membrane (or membrane deformability).[72] Among these factors, cell geometry as determined by the contribution of the surface-to-volume ratio is the most important, as exemplified by the cellular lesion of hereditary spherocytes. On the other hand, the intrinsic viscoelastic properties of the red cell are likely to have a relatively small effect on red cell survival. Southeast Asian ovalocytes are very rigid, yet they have a normal survival in vivo.

The cellular geometry, i.e., the biconcave disc shape of red cells, is critical for their survival. This cell surface shape provides a high ratio of surface area to cellular volume. The normal volume of the erythrocyte is about 90 μm^3; the minimum surface area that could encase this volume would be a sphere of about 98 μm^3. The surface area of a biconcave disc enclosing this volume is about 140 μm^3. Thus, shape alone provides the red cell with a considerable amount of redundant membrane and cytoskeleton. This feature provides the extra membrane surface area needed when red cells swell. More importantly, this geometric arrangement allows red cells to be stretched as they undergo deformation and distortion in response to the mechanical stress of the circulation. Loss of membrane by partial phagocytosis in immune hemolytic anemias or by fragmentation of bits of membrane from the cell in patients with cytoskeletal defects leads to elliptocytic or spherocytic shapes having greatly reduced surface area and, therefore, much less deformability.[73] The consequent reduction in tolerance of these cells to osmotic stress explains why anemias due to membrane defects are often accompanied by osmotic fragility, the basis for the clinical laboratory test. Conversely, if erythrocytes are engorged with water, they become macrospherocytic and less deformable.

Thus it is obvious that the organization of the membrane skeleton and its attachment to the plasma membrane influence the stability and deformability of the red cell. In the resting state, the folded helical segments of spectrin are highly coiled. Membrane deformation is accompanied by a rearrangement of the spectrin-actin-based membrane skeleton network with some spectrin molecules becoming uncoiled and extended, whereas others become more compressed and folded, resulting in no net change in surface area. Thus, shape changes but surface area does not. The extent to which this stretching and compression are possible determines the extent of deformability. Mutations or acquired alterations in membrane proteins that influence the spectrin-actin-based lattice of proteins leads to membrane loss with a concomitant decrease in surface area and a change in cell geometry.

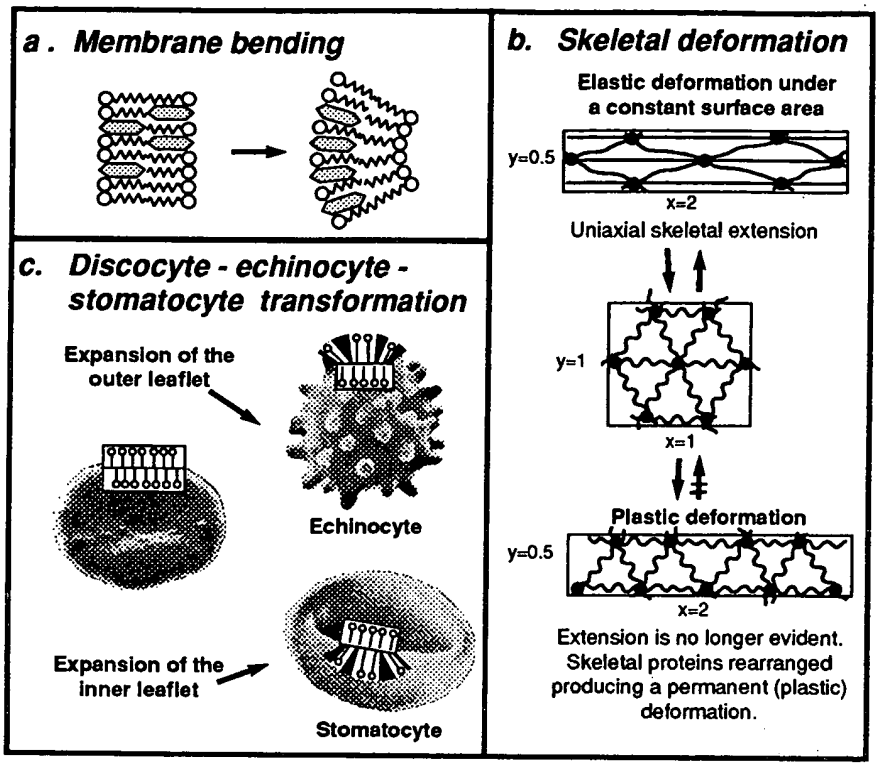

FIGURE 27-3 Material properties of the red cell membrane. *(a) Membrane bending.* The degree of membrane bending is restricted by the limited compressibility of the lipid bilayer. The rapid translocation of cholesterol (shaded diamonds) from the inner to the outer leaflet reduces the compression of the inner bilayer leaflet, thereby facilitating bending. *(b) Skeletal deformation.* While hydrophobicity of the red cell membrane lipid bilayer precludes the increase in its surface area without rupture, the membrane can undergo a large deformation under a constant surface area because of the viscoelastic properties of the membrane skeleton. During uniaxial extension, the skeleton undergoes stretching (*top rectangle*). After a cessation of an external force, a square surface area is resumed because the protein connections within this elastic skeletal network remain intact. Extensive or prolonged uniaxial extension leads to a rearrangement of the skeletal network because of a disruption of existing skeletal protein connections and a formation of new protein contacts. This leads to a permanent plastic deformation (*bottom rectangle*). *(c) Bilayer couple hypothesis and the stomatocyte-discocyte-echinocyte transformation.* Red cell shape reflects the ratio of the surface areas of the two hemileaflets of the lipid bilayer. The compounds (*black triangles*) that preferentially intercalate into the outer hemileaflet of the lipid bilayer produce its expansion followed by red cell crenation (echinocytosis or acanthocytosis). In contrast, expansion of the inner lipid bilayer leaflet produces a cup shape (stomatocytosis) and surface invaginations.

Red cell viscosity is largely determined by hemoglobin content.[73] At normal intracellular concentrations (27–35g/dl), viscosity contributes very little to cellular deformability. When erythrocytes become dehydrated, the effective intracellular hemoglobin concentration rises, and viscosity increases exponentially. Membrane pumps and channels normally maintain intracellular volumes that hold hemoglobin concentrations below the level at which cytoplasmic viscosity has an impact on deformability. Inherited anomalies of pumps or channels (e.g., hereditary xerocytosis) or derangements caused by polymerized or crystallized hemoglobin (e.g., sickle cell anemia or HbC disease), lead to cellular dehydration and greatly increased red cell viscosity.

MEMBRANE MATERIAL PROPERTIES

The material properties of the membrane reflect the properties of both the lipid bilayer and the skeleton. During deformation, the membrane

undergoes bending, which is restricted by the incompressibility of the lipid bilayer. It has been proposed that such bending is facilitated by the rapid translocation of cholesterol from the inner to the outer hemileaflet (Fig. 27-3). When red cells are suspended in hypotonic solutions, such as during osmotic fragility testing (see Chap. 43), they swell, reaching a nearly spherical shape because the bilayer membrane cannot expand its surface area more than 3 to 4 percent. Further lowering of the osmotic pressure results in membrane rupture, and intracellular hemoglobin is discharged into the supernatant.

The membrane skeleton determines both the solid and semisolid properties of the membrane. The solid properties are exemplified by an elastic extension of cells that completely restores their normal shape after the applied force has been removed. An example is a cell that has been deformed when passing through fenestrations of the splenic sinus wall. This elastic recovery of the normal shape is facilitated by the unique molecular anatomy of the skeletal lattice. Here the individual hexagons are in a compact, unextended configuration with the junctional complexes close to each other and the cross-linking arms of spectrin tetramers folded between them, thus allowing large unidirectional extensions without disruption of the lattice (see Fig. 27-3). The skeleton remains unperturbed during such deformation. On the other hand, application of large or prolonged forces allows the skeletal elements to reorganize into a new configuration; this produces a permanent plastic deformation. When the force is excessive, membrane fragmentation ensues. An example is vessels damaged when red cells are trapped by fibrin strands; after release from this site, the erythrocytes either are permanently deformed or are fragmented.

MEMBRANE BIOGENESIS AND AGING

Membrane protein biosynthesis occurs asynchronously during erythropoiesis. Early in erythroid development, the major proteins of the membrane skeleton (spectrin, ankyrin, and the 4.1 protein) are synthesized.[74,75] However, they turn over rapidly and do not assemble into a permanent network. At the proerythroblast stage, the synthesis of band 3 is initiated and, together with the synthesis of protein 4.1, increases up to the late erythroblast stage. During this time, mRNA levels and synthesis of spectrin and ankyrin protein decline. In contrast, the fraction of newly assembled spectrin and ankyrin protein on the membrane progressively increases, and the turnover of these proteins on the membrane declines.

Increased recruitment and stabilization of spectrin and ankyrin on the membrane in spite of the declining synthesis of these proteins is temporally related to a progressive increase in the synthesis of band 3 and protein 4.1, the principal bilayer anchors of the membrane skeleton.[76] Thus early studies suggested that the early steps of red cell membrane assembly were controlled by band 3 production where, after insertion into the membrane, it directed the assembly of stable macromolecular complexes from presynthesized pools of other proteins.[77,78] The role of band 3 in membrane assembly has been questioned by the following recent findings: (1) The organization of preformed pools of cytoskeletal elements induced by band 3 synthesis is not seen in nontransformed cells; and (2) band 3 knock-out mice exhibit normal membrane biogenesis even though their red cell membranes are unstable in the circulation.[79,80]

The biosynthesis and assembly of spectrin subunits is complex. β-spectrin biosynthesis exceeds that of α spectrin in the early erythroblasts derived from both embryonic (yolk sac) and fetal/adult (liver/spleen) origins. This ratio is preserved during later stages of erythropoiesis in embryonic cells, but not in fetal/adult-derived late erythroblasts and reticulocytes. In these latter cells, α-spectrin gene expression increases, whereas β-spectrin gene expression remains constant, resulting in a predominance of α-spectrin mRNA and protein during the late stages, when active assembly of the actual membrane is occurring most rapidly. $\alpha\beta$-spectrin subunits are incorporated into the membrane in a 1:1 stoichiometric ratio, regardless of their rates of synthesis.[74,81,82] This point is important in the analysis of inherited hemolytic anemias. Human α-spectrin synthesis exceeds that of β-spectrin by 2:1 during the later stages of erythropoiesis, when, presumably, membrane assembly is proceeding rapidly. The availability of β-spectrin subunits therefore determines the maximum rate and amount of stable spectrin assembly. Thus, mutations reducing steady-state levels of newly synthesized β spectrin should have a far greater phenotypic impact than do mutations causing comparable decreases in α-spectrin biosynthesis. Analyses of patients with hereditary hemolytic anemias support this prediction (see Chap. 43).

At the stage of orthochromatic erythroblast, when membrane biogenesis is nearly completed, the cell membrane undergoes a series of critical remodeling steps.[83,84] The membrane surrounding the nucleus contains an actin ring that likely participates in the expulsion of the nucleus from the erythroblast.[85] At the same time, the spectrin skeleton segregates into the region of the incipient reticulocyte, while some surface receptors cluster in membrane regions surrounding the extruded nucleus.

Some synthesis of spectrin, band 3, protein 4.1, and GPC continues in the newly enucleated reticulocyte, but most membrane remodeling occurs after translation. The reticulocyte is multilobular and motile; it possesses mitochondria, polyribosomes, and numerous membrane proteins that are either absent or much less abundant in mature red cells. In addition, phospholipid composition and inside-outside lipid distribution are different. Reticulocytes are far less deformable and considerably more unstable mechanically than are mature erythrocytes. Maturation begins in the bone marrow and lasts for 2 or 3 days. It is completed in the circulation and perhaps in the spleen where it has been termed *splenic polishing*. Reticulocytes first become cup-shaped before acquiring their final biconcave disc shape. This process involves major reorganization of both membrane phospholipids and cytoskeletal and embedded proteins, as well as the loss of lipids and proteins, including receptors for transferrin, insulin, and fibronectin.

RED CELL AGING

The mechanism of red cell aging is discussed in Chap. 29.

FETAL RED CELLS

Fetal erythrocytes differ in a number of respects including activity of both glycolytic and nonglycolytic enzymes, altered ATP and phosphate metabolism, differences in methemoglobin content and oxygen affinity, and altered storage characteristics (reviewed in Gallagher[86]). These erythrocytes exhibit increased rigidity, increased mechanical fragility, and decreased life span (average 45 to 70 days) compared to adult red cells.

There are also differences in the membranes of fetal and adult erythrocytes. ABO and I antigens and the receptors for the adsorbed serum antigens of the Lewis system are incompletely expressed. Fetal membranes are more permeable to monovalent cations and contain less Na^+-K^+-ATPase activity. They contain more phospholipid and cholesterol per cell and, as a consequence, have a larger surface-to-volume ratio and are slightly more osmotically resistant than adult cells. The ratio of sphingomyelin to phosphatidylcholine is increased in fetal membranes and differences in fatty acid composition exist, but these changes evidently tend to balance each other, as membrane fluidity is normal. The protein composition of fetal red cell membrane is quantitatively normal.

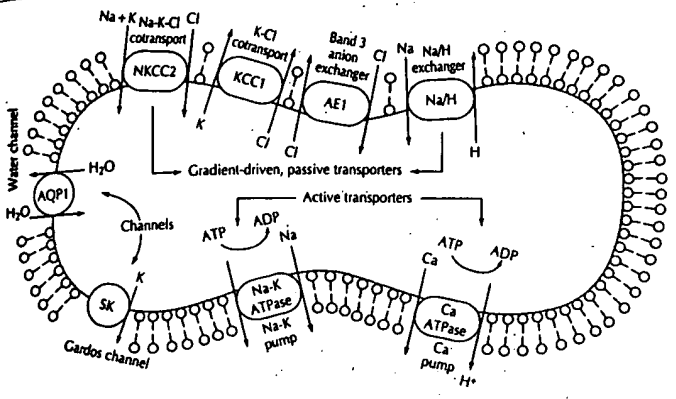

FIGURE 27-4 Principal ion transport pathways of the human erythrocyte. AE-1: band 3, the anion exchanger; AQP1: the water channel aquaporin 1; KCC-1: KCl cotransport system of the family of chloride-cation cotransporters; NKCC2: basolateral molecular form of Na-K-Cl cotransport; SK: small conductance potassium channel. Reprinted with permission from Brugnara.[87]

MEMBRANE PERMEABILITY

The normal red cell membrane is nearly impermeable to monovalent and divalent cations, thereby maintaining a high potassium, low sodium, and very low calcium content. In contrast, the red cell is highly permeable to water and anions, which are readily exchanged, and as a result erythrocytes behave as nearly perfect osmometers. Water and ion transport pathways in the red cell membrane (Fig. 27-4) include energy-driven membrane pumps, gradient-driven systems, and various channels.[87,88] An important feature of the normal red cell is its ability to maintain a constant volume. The mechanisms by which red cells "sense" changes in cell volume and activate appropriate volume regulatory pathways are unknown. Glucose is transported without the expenditure of energy utilizing a transporter, while larger charged molecules, such as ATP and related compounds, do not cross the normal red cell membrane, although phosphoenolpyruvate is an exception to this rule (Chap. 140).

The effects of disruption of the red cell permeability barrier are illustrated by complement-mediated hemolysis. Complement activation on the red cell surface leads to formation of the membrane attack complex, composed of terminal complement components embedded in the lipid bilayer. This multimolecular complex acts as a cation channel, allowing passive movements of sodium, potassium, and calcium across the membrane according to their concentration gradients. Attracted by fixed anions, such as hemoglobin, ATP, and 2,3-BPG, sodium accumulates in the cell in excess of potassium loss and in excess of the compensatory efforts of the Na^+/K^+-pump. The resulting increase in intracellular monovalent cations and water is followed by cell swelling and ultimately colloid osmotic hemolysis.

ENERGY-DRIVEN MEMBRANE PUMPS

In the red cell, two ion-motive ATPase-dependent cation pumps maintain low intracellular sodium and calcium and high potassium.[87] The ouabain inhibitable Na^+-K^+-ATPase (the sodium pump) extrudes sodium in exchange for potassium in a 3:2 stoichiometry. Ca^{++}-ATPase is a calmodulin-activated pump that extrudes calcium from the red cell and maintains a very low intracellular calcium concentration, thus protecting cells from multiple deleterious effects of calcium. Examples of such deleterious effects include echinocytosis, membrane vesiculation, calpain activation, membrane proteolysis, and cellular dehydration. Elevated intracellular calcium plays an important role in the pathophysiology of sickle cell disease, as increased levels of intracellular calcium observed during sickling are due to an increase in Ca^{++} flux and reduced activity of the Ca^{++}-ATPase. The membrane also contains an ATP-driven GSSG transporter (Chap. 26) and amino acid transport systems.

GRADIENT-DRIVEN SYSTEMS

The Na^+/K^+ gradient established by the sodium pump is used by several passive, gradient-driven systems to move ions across the red cell membrane.[87] These include the K^+Cl^+-cotransporter, band 3 (see above), the Na^+-K^+Cl-cotransporter, and the Na^+-H^+-exchanger. The Na^+-K^+Cl-cotransporter plays only a minor role in the red cell. The Na^+-H^+-exchanger appears to play a role primarily in early erythrocyte maturation. The K^+Cl^+-cotransporter is a typical carrier-mediated cotransporter, which is particularly active in reticulocytes.[89,90] It is activated by cell swelling, acidification, depletion of intracellular magnesium and thiol oxidation.

CHANNELS

Channels of the red cell include voltage-gated channels (mediated via Na^+K^+-ATPase), water channels (the aquaporins), and the Ca^{++}-activated K^+-channel. The Ca^{++}-activated K^+-channel, also called the *Gardos channel* after its discoverer Dr. George Gardos, causes selective loss of K^+ in response to an increase in intracellular $Ca.^{++}$[91,92] In sickle cells, increased activity of both the Gardos channel and the K^+-Cl-cotransporter leads to a net loss of K^+ and water, leading to cellular dehydration and the formation of intermediate and hyperdense erythrocytes.[93,94] Recently, pharmacologic manipulation of these two channels has been tried in attempts to improve cellular hydration of the red cell and ameliorate the clinical course of patients with sickle cell disease.[95,96]

The aquaporins are membrane channel proteins that serve as selective pores through which water crosses the plasma membrane.[97,98] Aquaporin-1, AQP1, which is expressed in many tissues including erythrocytes, contributes to the ability of the red cell to adjust rapidly to changes in osmolality. AQP1 contains the epitope for the Colton blood group system. The genetic basis of the rare Colton null phenotype has been identified as a mutation of the highly conserved NPA motif of AQP1 essential for channel function.[99] Colton null individuals exhibit no obvious clinical phenotype, although mice with targeted inactivation of AQP1 become hyperosmolar after fluid restriction.[100] Recently, evidence for the presence of AQP3 in erythrocytes has been presented.[101]

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